Construction and use of a *Sox1* reporter cell line to study embryonic stem cell differentiation

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I declare that the work described in this Thesis is my own, except where otherwise stated

Marios Pericles Stavridis

To my parents

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Abstract

One of the biggest challenges in embryonic stem (ES) cell biology is to direct ES cell differentiation towards a specific cell type. So far, this has not been achieved in any meaningful way. Therefore, the alternative method of selecting desirable cell types from a mixed pool of cells at different stages of commitment and differentiation remains the best option for obtaining pure populations. Several methods have previously been employed for this purpose, including immunopurification, selective viability or attachment, use of growth factors selectively to amplify desired populations as well as lineage selection based on the expression of a gene conferring drug resistance from an endogenous tissue restricted promoter.

Sox1 is member of the group B of the Sox family of transcription factors, expressed during embryonic development in mammals. It is expressed throughout the neural axis from the headfolds stage, and also in the lens. *Sox1* is upregulated during ES cell differentiation into neural precursors, and its misexpression causes an EC cell line to differentiate into neurons. *Sox1* expression during nervous system development is associated with proliferating cells of the CNS, expression being lost as cells exit mitosis and terminally differentiate. Its expression pattern is more restricted than that of most other markers for early neural cells such as nestin, making it a good marker gene for the study of neural development both *in vivo* and *in vitro* from ES cells.

Here I have used gene targeting to generate a reporter ES cell line (46C) in which the *Sox1* open reading frame is replaced by the gene encoding enhanced green fluorescent protein linked to a selectable marker. The use of EGFP enables the observation of *Sox1* expression in live cells. The expression of the reporter faithfully recapitulates the normal expression of *Sox1 in vivo* in mice generated from the 46C cells. This cell line has been used to analyse the differentiation of ES cells to neural fates, in particular to characterise a newly discovered, monoculture differentiation system. *Sox1*-EGFP expression is monitored by flow cytometry, which enables quantification of the differentiation process. The effect of proteins and inhibitors implicated in neural determination has been monitored quantitatively and over time using this system. Acquisition of neural fate occurs rapidly in the absence

of any inducers or serum, and without formation of multicellular aggregates. BMP-4 completely blocks this neural fate specification, similarly to the situation in the frog and the chick. Collectively, the results indicate that restriction of ES cell pluripotency to neural fates occurs in a manner resembling default neural induction in amphibians.

1 Introduction

1.1 Embryonic stem cells

Mouse embryonic stem cells (ES cells) are cell lines derived from the pluripotent compartment of early embryos, the inner cell mass of the blastocyst (Evans and Kaufman, 1981; Martin, 1981). A blastocyst is composed of an inner cell mass component and a layer of trophectoderm externally surrounding the inner cell mass and a cavity, the blastocoel. The inner cell mass further differentiates into the hypoblast and the epiblast. The hypoblast will give rise to parietal and visceral endoderm, both of which contribute to the formation of the yolk sac; the epiblast gives rise to the embryo proper (Hogan *et al.*, 1994). Culture of blastocyst stage embryos *in vitro* under suitable conditions in the presence of serum gives rise to an outgrowth which can be dissaggregated and replated, producing several types of secondary colonies, including some with a characteristic undifferentiated morphology. These colonies can be expanded and produce ES cell lines capable of continuous propagation and clonal expansion (Robertson, 1987a). ES cells are thought to represent the epiblast component of the inner cell mass (Brook and Gardner, 1997).

The derivation and maintenance of undifferentiated ES cells was originally dependent on culture on feeder layers of mitotically inactivated mouse embryonic fibroblasts (Evans and Kaufman, 1981; Martin, 1981), or in suitably conditioned medium (Smith and Hooper, 1987). In the absence of such factors, ES cells rapidly lose their undifferentiated phenotype and undergo extensive and irreversible differentiation to mesodermal and endodermal cells, implying that maintenance of the undifferentiated phenotype is dependent upon active suppression of differentiation by factor(s) made by the feeders (Smith and Hooper, 1987). One such factor was identified as the IL-6 family cytokine leukaemia inhibitory factor (LIF) (Gearing *et al.*, 1988; Smith *et al.*, 1988; Williams *et al.*, 1988). LIF and a small group of other related cytokines that act through the gp130 receptor can alleviate the requirement for feeders in the propagation of ES cell lines (Nichols *et al.*, 1990; Yoshida *et al.*, 1994). The processes downstream of gp130 responsible for the maintenance of the undifferentiated phenotype depend on the activation of the transcription factor STAT3 (Burdon *et al.*, 1999; Matsuda *et al.*, 1999; Niwa *et al.*,

1998). Maintenance of undifferentiated ES cells is also dependent on precise levels of expression of the transcription factor Oct4 (Nichols *et al.*, 1998; Niwa *et al.*, 2000). Embryos lacking Oct4 form blastocysts lacking any pluripotent cells. Instead, the inner cell mass can only give rise to extraembryonic trophoblast (Nichols et al., 1998). Forced overexpression of *Oct4* in ES cells causes differentiation into primitive endoderm and mesoderm, while repression of Oct4 expression causes trophectoderm differentiation (Niwa et al., 2000).

Self-renewing ES cells are pluripotent like the inner cell mass cells from which they are derived. This capacity is demonstrated *in vitro* by the differentiation into a plethora of different cell types (see below), and *in vivo* upon re-introduction into a blastocyst whereby they contribute to all foetal and adult lineages (including the germ cell lineage) plus yolk sac mesoderm, allantois and amnion of chimeras (Beddington and Robertson, 1989; Bradley *et al.*, 1984). Therefore, ES cells provide a model for the study of cell commitment and differentiation as well as providing a means to dissect normal development via introduction of specific mutations into the genome. The discovery of primate (Thomson *et al.*, 1995; Thomson and Marshall, 1998) and subsequently human multipotent cell lines (Reubinoff *et al.*, 2000; Shamblott *et al.*, 1998; Thomson *et al.*, 1998) spurred a new interest in ES cell biology, the potential use of stem cell derived material in cell replacement therapies for diseased or damaged tissue (Smith, 1998; Svendsen and Smith, 1999). Recent findings (Reubinoff *et al.*, 2001; Zhang *et al.*, 2001) indicating that such a goal may be attainable for Parkinsonian degeneration have further fuelled interest in ES cells and their differentiation.

An exciting prospect for the use of ES cell derived material for cell replacement therapy is the use of "customised" ES cell lines to avoid immune response of the host to the donor cells. This can be achieved by producing an ES cell line by nuclear transfer of host nucleus to an unfertilised egg, culture of the resulting embryo to the blastocyst stage and derivation of ES cells, which are entirely derived from the host-to-be. Experiments with mice have shown that this two-step procedure is feasible (Hochedlinger and Jaenisch, 2002; Kawase *et al.*, 2000; Munsie *et al.*, 2000; Wakayama *et al.*, 2001).

1.2 Neural induction

The vertebrate central nervous system originates from a part of the embryonic ectoderm, the neural plate. Primitive ectoderm derived from the epiblast during gastrulation gives rise to both the neural plate and the epidermis depending on the nature of the signals it receives based on its location. Neuralising signals emanate from a region known in vertebrates as the organiser (also called Spemann's organiser in frogs, Hensen's node in the chick and the node in the mouse) which appears at the onset of gastrulation. Transplantation of the organiser to an ectopic location in the ectoderm of a host embryo normally destined to give rise to epidermis causes the induction of a secondary axis the neural tissues of which are derived from the ectodermal cells of the host, while the transplanted donor tissue makes mesodermal structures (notochord and pre-chordal mesoderm) (Hogan *et al.*, 1992).

In the mouse, patterning of anterior nervous system requires another tissue, the anterior visceral endoderm (AVE)(Thomas and Beddington, 1996), often called the head organiser. The AVE can be distinguished molecularly by the expression of several genes (*Hex, Lim1, goosecoid, Otx2, goosecoid, cerberus-related 1* and *Hesx1*)(Beddington and Robertson, 1999). It also produces Nodal, a TGFβ-like molecule necessary for the establishment of anteroposterior polarity (Brennan *et al.*, 2001). AVE is required for patterning the anterior of the embryo together with the epiblast as shown by transplantation experiments, but is not sufficient to induce ectopic neural tissue (Tam and Steiner, 1999).

The role of the organiser however is not to directly induce neural tissue, since dissociation of naïve frog ectoderm is sufficient to cause neural induction in the absence of organiser (Grunz and Tacke, 1989). It is now known that the organiser participates in the differentiation between neural and non-neural (epidermal) ectoderm by antagonising the action of bone morphogenetic proteins (BMPs) (Wilson and Hemmati-Brivanlou, 1995).

BMPs are members of the transforming growth factor beta (TGFβ) family of secreted factors. During gastrulation in frogs, naïve ectoderm cells express BMP4 and BMP7. The organiser tissue (dorsal mesoderm) that forms during gastrulation expresses secreted proteins capable of binding and inactivating BMPs (Noggin, Chordin and Follistatin) (Hemmati-Brivanlou *et al.*, 1994; Piccolo *et al.*, 1996;

Zimmerman *et al.*, 1996). The effect of inactivation of BMP signals by antagonists produced by the organiser is that naïve ectoderm becomes neural. In the presence of BMP signals, when for example naïve ectoderm is explanted and cultured in BMPs, ectoderm becomes epidermal. These results, derived mainly from work in the frog *Xenopus laevis* gave rise to the so-called "default model" for neural induction: ectoderm is fated to become neural unless BMP signals change its fate to epidermis (Hemmati-Brivanlou and Melton, 1997) (Figure 1.1A).

A conflicting view of neural development is that neural induction starts before gastrulation (and therefore before the organiser is present to antagonise BMP activity), and that FGF signalling is required for it (Hongo *et al.*, 1999; Launay *et al.*, 1996; Streit *et al.*, 2000; Wilson and Edlund, 2001). This view is strengthened by the observation that neural tissues can develop in the mouse in the apparent absence of a node (Klingensmith *et al.*, 1999), or even in the absence of two BMP antagonists (Noggin and Chordin) (Bachiller *et al.*, 2000). In the chick, BMP antagonists are unable to impart neural fate on primitive ectoderm or lateral epiblast (Streit *et al.*, 1998; Wilson *et al.*, 2000), indicating that neural induction involves some other factor(s), possibly acting in conjunction with BMP antagonists. A strong candidate for such a factor is FGF.

In chick ectodermal explants FGF receptor antagonists can block neural induction (Streit et al., 2000; Wilson *et al.*, 2001). This can be rescued by BMP antagonists if a low concentration of the FGF antagonist is used, but in the presence of high FGF antagonist concentration the block is irreversible by BMP inhibition (Wilson et al., 2001). Some evidence indicates that a role of FGF signalling is to repress *Bmp* at the transcriptional level (Wilson et al., 2001), and that FGFs have a dual role in neural induction: high concentrations of FGF repress *Bmp* expression, whereas lower levels of FGF can act via an unknown, independent mechanism to promote neural identity.

Wnt signalling is also believed to be important for neural induction, as injection of Wnt mRNA into early *Xenopus* embryos causes formation of ectopic neural tissue (Baker *et al.*, 1999). However, at later stages of development Wnt signals inhibit neural induction (Baker et al., 1999; Wilson et al., 2001). There is evidence (Wilson et al., 2001) that the inability of BMP antagonists and FGFs to promote neural fate in prospective epidermal cells in chick is due to Wnt signalling

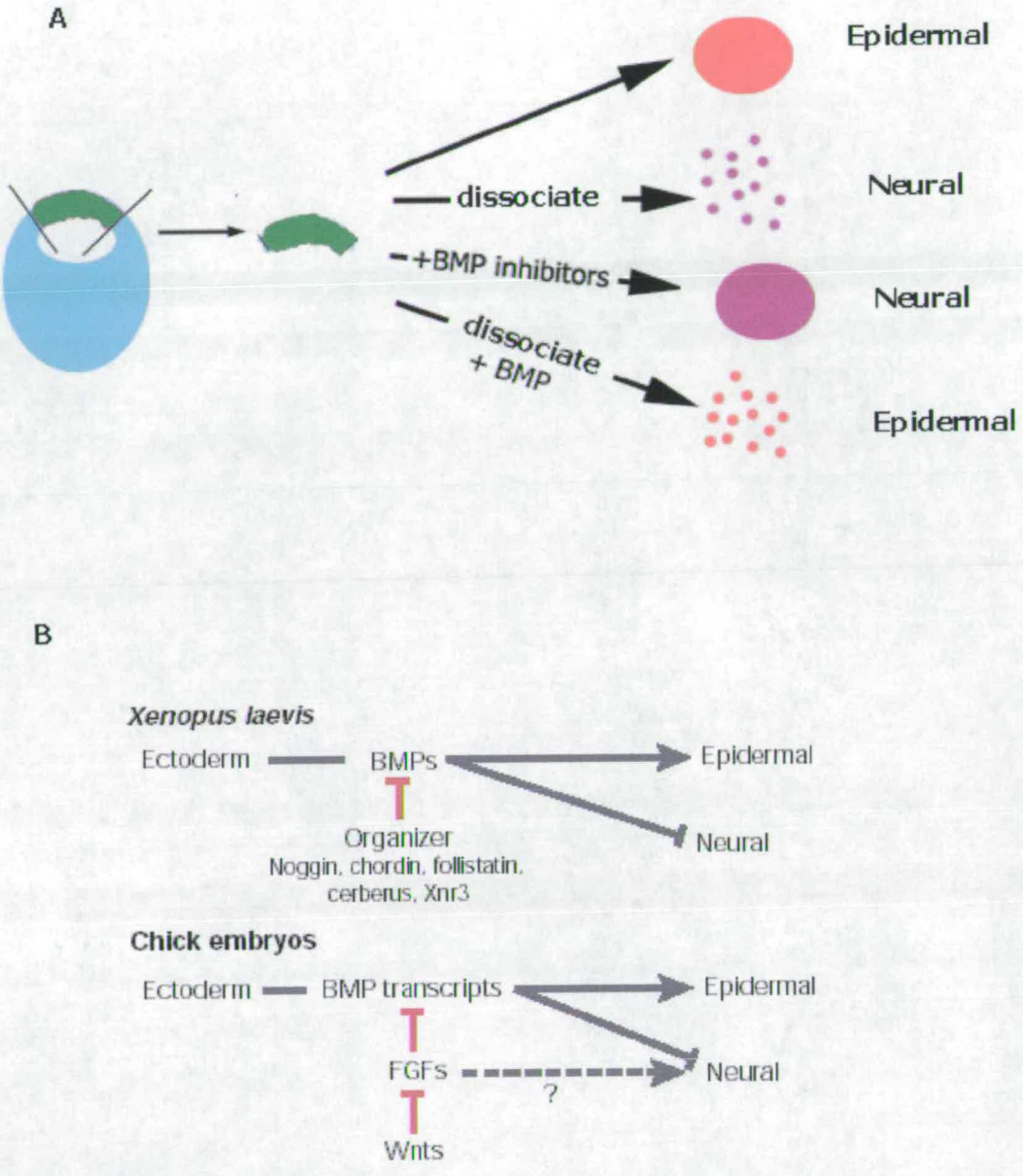


Figure 1.1 The default model for neural induction

A: The fate of *Xenopus laevis* animal cap explants is determined by the culture conditions and the presence of active BMP signalling. B: Schematic indicating the differences between neural induction in *Xenopus* and chick (Munoz-Sanjuan and Brivanlou, 2002).

present in such tissues. High levels of Wnt may inhibit the BMP-independent FGF transduction pathway required for neural differentiation in prospective epidermis, while absence of Wnt in prospective neuroectoderm allow FGFs and BMP antagonists to neuralise it (Wilson et al., 2001) (Figure 1.1B).

Interestingly, cells from the organiser and organiser derivatives express not only BMP antagonists, but also Wnt antagonists and FGFs (Glinka *et al.*, 1998; Pera and De Robertis, 2000; Shibata *et al.*, 2000), which is consistent with this model. If Wnt antagonism and FGF signalling are required for neural induction, an organiser graft will provide those as well as BMP antagonists while inducing an ectopic nervous system. Therefore, the alternative model for neural induction states that Wnt is initially excluded from the prospective neuroectoderm, permitting FGFs and BMP antagonists to act and induce neural tissue, whereas in prospective epidermis, Wnt and BMP signals synergise to impart epidermal fate (Wilson and Edlund, 2001). A common observation in all of these results, in all vertebrates, is that neural induction is negatively regulated by BMP signals. This may be seen as evidence that the underlying mechanism is similar.

1.3 Neural differentiation of ES cells

As mentioned previously, removal of feeders and LIF causes ES cell differentiation. In such differentiating cultures, mesodermal and endodermal markers are expressed, albeit in a disorganised and ill-defined manner (Smith, 2001). However, more organised differentiation can be achieved by several methods. A variety of different cell types have been produced from ES cells, often with simple modifications in the differentiation protocols (Dani *et al.*, 1997; Guan *et al.*, 1999; Wiles and Keller, 1991).

To date, the best-studied mode of ES cell differentiation is the formation of embryoid bodies (EBs) (Doetschman *et al.*, 1985). This method for differentiation of stem cells has its origin in the study of embryonal carcinoma (EC) cells (Martin and Evans, 1975; Martin *et al.*, 1977) and involves the formation of multicellular cell aggregates in suspension culture. Within these aggregates, complex interactions between heterologous cell types result in the induction of differentiation of stem

cells to derivatives of all three embryonic germ layers (Martin et al., 1977). Plating of the embryoid bodies causes further differentiation and outgrowth.

Such differentiation invariably gives rise to multiple cell types (often of all three germ layers) and despite its merits in the study of cell commitment and differentiation, has little value in the production of cell types for transplantation-based cell therapy. More homogenous differentiation can be achieved by addition of morphogens, use of selective media and plating on different substrata (Dani et al., 1997; Guan et al., 1999; Okabe *et al.*, 1996; Rohwedel *et al.*, 1999; Wiles and Keller, 1991). Retinoic acid is one such example of a morphogen; it is produced by Hensen's node (Hogan et al., 1992) and has been shown to be a strong posteriorising agent (Kessel and Gruss, 1991) for the whole embryonic axis, including the nervous system (Marshall *et al.*, 1992).

Retinoic acid gradients arise by the synthesis and breakdown of retinoic acid by the enzymes RALDH (retinaldehyde dehydrogenase) and CYP26 respectively. RA synthesis is occurring (apart from the node) in the posterior mesoderm and later the somites, optic vesicles, the gut and also in the differentiating limbs (Niederreither *et al.*, 1997). RA breakdown is responsible for the correct level of RA (and consequently patterning) of the hindbrain, the vertebrae and the tailbud, with abnormal development of these structures in mice lacking CYP26 (Sakai *et al.*, 2001). Retinoic acid synthesis is not necessary for neural induction, but RA deficient embryos have defects in neural patterning including open neural tube, anterior truncations and irregular neural tube folding. At least some of these defects may be caused by abnormal *Fgf3* gene expression in the hindbrain (Niederreither *et al.*, 1999). Retinoic acid has complex effects on embryoid bodies; depending on the time of application, the concentration and the duration of the culture it can induce widely varied cell types (Rohwedel et al., 1999).

1.3.1 Neural differentiation in embryoid bodies

The generation of ES cell-derived neural cells, especially neurons (Bain, 1995; Fraichard *et al.*, 1995; Strubing *et al.*, 1995) is a well-studied example of ES cell differentiation. Neural differentiation of ES cells has been achieved by several different protocols, some of which are strikingly different. Initial insight was provided again by EC cells; P19 EC cells readily differentiate into neurons by simple

aggregation and retinoic acid treatment (Jones-Villeneuve *et al.*, 1982; McBurney, 1993). The first protocols for neural differentiation of ES cells were based on the P19 differentiation protocol; embryoid bodies were treated with retinoic acid at different time windows and then plated onto laminin (Bain, 1995), gelatin (Strubing et al., 1995) or tissue culture plastic (Fraichard et al., 1995).

Cells with overt neuronal morphology appeared after plating, and were found to express neuronal specific genes such as neurofilament light chain (NF-L), microtubule associated proteins (MAP) 2 and 5, synaptophysin and others. These cells were found to respond to a range of neurotransmitters and depolarising currents, confirming that they were indeed excitable neurons. Glial cell types also appeared in such differentiated cultures, as judged both by morphology and expression of specific glial markers. The majority of glial cells produced were astrocytes, but oligodendrocytes have been generated and selectively expanded from EB cultures (Liu *et al.*, 2000).

A variant protocol for neural differentiation of ES cells involved the formation of embryoid bodies without retinoic acid treatment, but depended on the subsequent culture of the attached EBs in a selective, serum-free medium to eliminate non-neural cells. Culture in that medium results in a dramatic decrease in cell number, but an enrichment of the culture in nestin (Lendahl *et al.*, 1990) positive neural progenitor cells which can be expanded and induced to differentiate into neurons with high efficiency (Okabe et al., 1996).

The majority of neurons generated following EB differentiation are GABAergic, with some glutamatergic and cholinergic neurons also generated (Bain, 1995; Fraichard et al., 1995; Mujtaba *et al.*, 1999; Okabe et al., 1996; Strubing et al., 1995). More recently it has been possible to instruct neural progenitors derived from ES cells to differentiate to dopaminergic and serotonergic neurons (Lee *et al.*, 2000b), present in the ventral midbrain. Dopaminergic neurons are those depleted in Parkinson's disease, a disease where ES cell-based replacement therapy may be particularly applicable (Deacon *et al.*, 1998; Dunnett *et al.*, 2001; Svendsen and Smith, 1999). This was achieved by addition of Sonic hedgehog and FGF-8 in the medium in which neural progenitors were being expanded. The rationale for this instructive mechanism came from experiments with neural plate explants (Ye *et al.*, 1998). Sonic hedgehog (Shh) is a protein secreted by the notochord and ventral

midline during patterning of the neural tube and the brain and has ventralising properties (Yamada *et al.*, 1993). FGF-8 is produced by a ring of cells at the isthmus, the midbrain-hindbrain border, and regulates the patterning and polarity of the midbrain (Crossley *et al.*, 1996). The fact that the neural precursors generated from ES cells can respond to morphogenetic cues suggests that they are (at least to some degree) plastic, and some parallels can be drawn between neural differentiation of ES cells and neural development *in vivo*.

FGF8 is also expressed in the limb apical ectodermal ridge, the primitive streak, tail bud, the branchial arches, the facial surface ectoderm and other regions known to direct outgrowth and patterning (Crossley and Martin, 1995). Targeted disruption of *Fgf8* causes failure of cell migration through the primitive streak and anterior movement of the AVE, resulting in defects in the patterning of the anterior ectoderm. A hypomorphic allele of *Fgf8* in homozygous state or combined with a null allele causes deletions of midbrain, hindbrain and olfactory bulbs as well as small forebrain alongside craniofacial and other non-neural defects (Meyers *et al.*, 1998). FGFR1 null embryos die early in development showing growth and mesodermal patterning defects. FGF receptor 1 (FGFR1) null: wildtype chimeras show an important role of FGF signalling in primitive streak crossing, resulting in ectopic neural tissue. This indicates a role of FGFs in the germ layer specification through morphogenetic movements at gastrulation (Ciruna *et al.*, 1997).

1.3.2 Stromal cells

A distinct system for differentiation of ES cells to dopaminergic neurons has been described (Kawasaki *et al.*, 2000). This system differs from the other methods for neural differentiation in that the differentiation process happens not in aggregates, but on adherent co-cultures of ES cells with bone marrow-derived stromal cells (PA-6) in serum-free medium. No retinoic acid is used to induce neural fate and no Shh or FGF-8 is added to the cultures to induce dopaminergic neurons. This is a good indication of an inductive mechanism for the production of neurons from ES cells, although the molecular nature of the inducing signal(s) remains elusive. Importantly, although this unknown activity (termed stromal cell-derived inducing activity or SDIA) can act weakly through a filter, it does not condition the medium and surprisingly, is not destroyed by fixation of the stromal cells. Other cell

types (e.g. fibroblasts) that do not normally exhibit SDIA can induce dopaminergic neuron generation from ES cells after fixation. This may indicate that the inductive signal(s) are negatively affected by the presence of surface and/or secreted proteins (e.g. LIF) in cell types other than PA-6. SDIA-mediated differentiation is suppressed in the presence of serum or BMP4. This raises the possibility that neural determination of these cells is due to a specific lack of signals inducing alternative differentiation, as postulated by the default model, and that the primary role for the stromal cells is to provide attachment to the ES cells. The dopaminergic phenotype of the neurons produced on PA-6 could be due to the presence of a factor produced by the cells.

1.3.3 Defined media

The first indication that ES cell neural determination is under inhibitory control and resembles "default" amphibian neural induction came by experiments in serum-free medium (Wiles and Johansson, 1999). Using embryoid bodies generated in chemically defined serum-free medium, this group showed that unless signals of the transforming growth factor superfamily (including BMPs and Activin) were present, some neural markers were expressed. In the presence of Activin A, an inducer of mesoderm in the frog, mesodermal as well as neural markers appeared, along with a strong induction of the Activin antagonist, Follistatin (Wiles and Johansson, 1999). BMP-4 treatment resulted in the loss of neural cell markers, similarly to the situation in frog ectodermal explants (Wilson and Hemmati-Brivanlou, 1995). BMP-4 also suppressed differentiation of ES cells in a related system (Tropepe *et al.*, 2001). In this case, ES cells were selected in serum-free conditions in the presence of LIF, and at a frequency of 0.2% gave rise to nestin-positive cell aggregates ("neurospheres") which could be then expanded in FGF-2 or induced to differentiate into neurons, astrocytes or oligodendrocytes. ES cells lacking components of the BMP signalling pathway gave rise to 3.5 fold more "neurospheres", suggesting that differentiation in this system is under inhibitory control by BMPs (Tropepe et al., 2001). The differentiation was inhibited by blocking antibodies to FGF-2, revealing a striking parallel to the situation in the chick, where FGF signalling seems to be necessary for neural differentiation of naïve ectodermal cells (Streit et al., 2000; Wilson et al., 2000). However, the plating efficiency under

the conditions described in that paper is very poor, raising the possibility that the effect of FGF-2 is to sustain viability of the initial cell population rather than to promote competence for neural induction.

Taken together, all the above examples of neural differentiation indicate that despite being an *in vitro* system, ES cell differentiation has many similarities to normal embryonic development, at least as far as neural induction is concerned.

1.3.4 Monolayer differentiation

A novel protocol for the differentiation of ES cells to the neural lineage in fully defined, serum-free monolayer culture was recently developed in our laboratory (Ying *et al.*, submitted). This protocol involves the plating of ES cells on gelatinised plastic in fully defined N2B27 medium (see materials and methods).

The cells convert efficiently into neural precursors that subsequently differentiate to neurons and glia in the absence of serum or added growth factors. After dissection of the components of N2B27, only transferrin was found essential for cell viability, whilst other proteins (i.e. albumin and insulin) assisted with cell attachment and survival but are dispensable for differentiation (Ying *et al.*, submitted). Under these serum-free conditions initial plating efficiency is lower than in more standard serum supplemented media, but subsequent viability is high and cell numbers increase exponentially throughout the differentiation process. The method is not due to selection or selective amplification of pre-differentiated cells in the cultures as for the first 2 days after plating, cells still express *Oct-4* and produce ES cells monolayers if culture medium is changed to ES cell medium with LIF (Ying *et al.*, submitted). Since the differentiation process is happening in adherent monolayer, it is possible to observe the morphological changes of the cells as they differentiate.

This system for neural differentiation of ES cells indicates that cellular interactions in multicellular aggregates are not necessary for the acquisition of neural fate. I have carried out a more detailed analysis of this system which is presented in chapter 5.

1.3.5 Enrichment strategies

As seen above, different groups use slightly different protocols in order to generate neural cells (Gottlieb and Huettner, 1999), and this often is dictated by the cell type desired. Since the available protocols almost invariably gave rise to a mixed population of neural and non-neural cells of varying stages of commitment, the stage following the initial differentiation often served the purpose of selecting the desired cells from the pool. This has been accomplished by a variety of methods. The selective viability in a minimal culture medium mentioned before (Okabe et al., 1996) is the first such example. Others include immunopanning for neural antigens to select neural- or glial- restricted progenitors (Mujtaba et al., 1999), a combination of growth factor stimulation and differential adhesion to obtain oligodendrocyte precursors (Liu et al., 2000), and the use of a selectable marker knock-in to select neural precursors (Li *et al.*, 1998).

This technique of lineage selection depends on the restriction of expression of the selected gene, but can yield pure populations of cells in a short time and following any differentiation protocol. A first application of it is based on the fact that proliferating neural progenitors arise during differentiation, and they express the early neural gene *Sox2*. A selectable marker (βgeo) introduced into the *Sox2* locus enables the purification of Sox2 expressing cells by addition of G418 during differentiation. Non-neural and post mitotic neural cells not expressing *Sox2* are killed by the drug resulting in a homogeneous population of *Sox2* positive cells. These cells can then be expanded in the presence of mitogens or left to differentiate to functional neurons and glia (Figure 1.2)

An important issue regarding the potential use of ES cell-derived material for transplantation in diseased or lesioned central nervous system (CNS) is the production of the correct neuronal class and the elimination of persistent ES cells, which usually cause teratocarcinomas when transplanted into an animal (Evans and Kaufman, 1983). In the case mentioned above, where the *Sox2* gene was used to drive selection, persistent ES cells could not be eliminated because they too express *Sox2*. A combination of positive (Sox2) and negative selection for an independently targeted ES specific gene (*Oct4*) can give the desired purity (Q-L Ying, J Kawaguchi *et al.*, unpublished), however it requires multiple rounds of targeting.

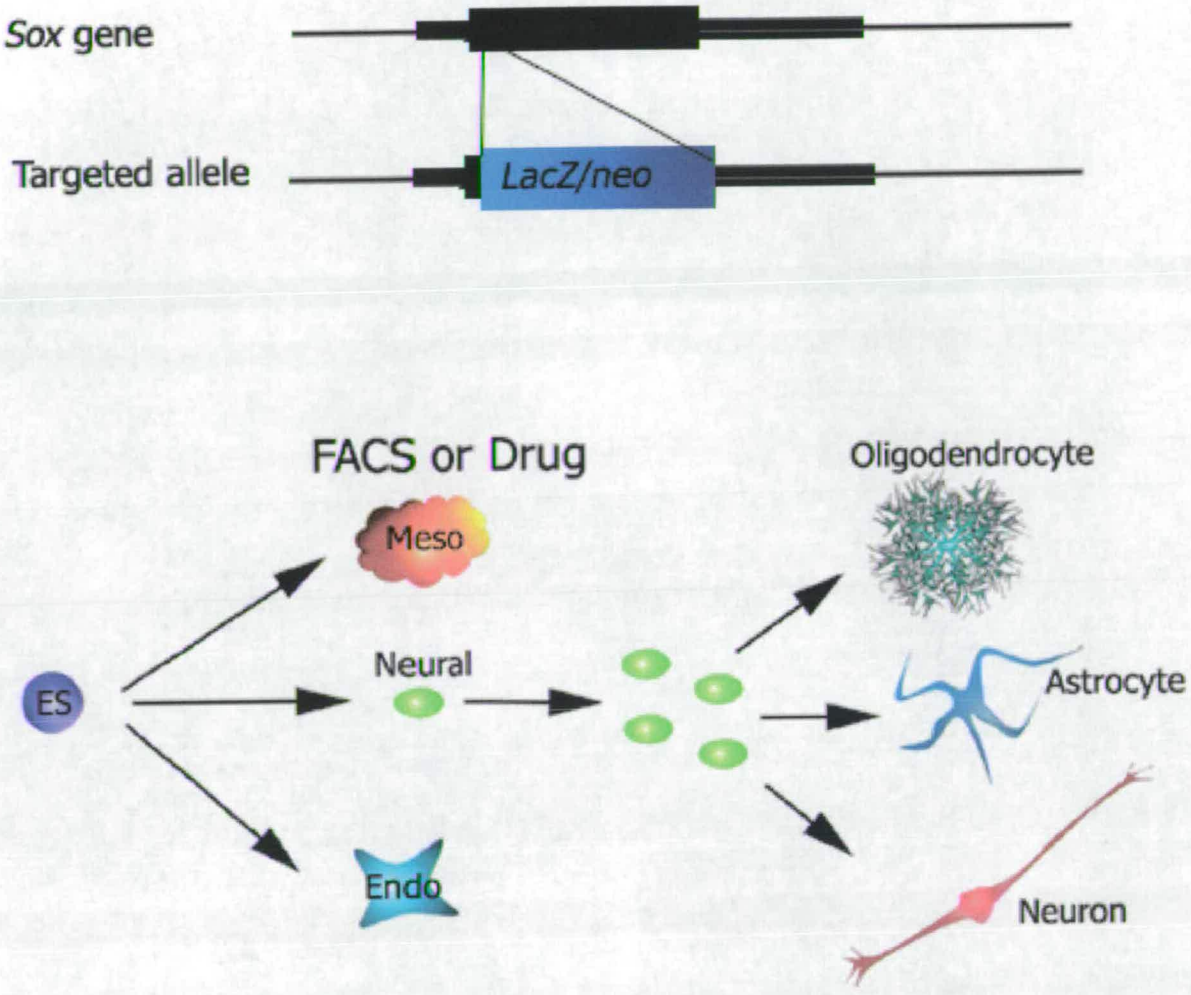


Figure 1.2 The principle of lineage selection as applied to ES cell neural differentiation

Top: gene targeting results in the insertion of a reporter/selectable marker into a *Sox* gene. Bottom: Following ES cell differentiation, cells expressing the reporter/selectable marker can be purified by flow cytometry or selective media and then induced to differentiate to neurons, astrocytes and oligodendrocytes. Figure adapted from Svendsen and Smith, 1999.

1.4 Sox genes

The Sox genes are a family of transcription factors identified by homology to the mammalian testis-determining gene, *Sry* (Gubbay *et al.*, 1990). There are now many members of the family found in organisms as diverse as human, rodents, amphibian, fly, worm and fish (reviewed in (Pevny and Lovell-Badge, 1997; Prior and Walter, 1996; Wegner, 1999). Their defining characteristic is the presence of a "Sry box", a high mobility group (HMG)-like DNA binding domain which gives the family its name. Sox family HMG boxes have at least 50% sequence identity with that of the founding member of the family, mouse *Sry* (Denny *et al.*, 1992; Pevny and Lovell-Badge, 1997). Sox genes are a large family of at least 24 members, and can be further categorised into groups according to the homology of their HMG boxes.

Members of the same group have more than 80% homologous HMG boxes, and often share homology even outside the HMG domain (Collignon *et al.*, 1996; Wegner, 1999). The HMG box of Sox proteins can bind DNA in a sequence specific fashion, a function shared only with members of the TCF/LEF family of HMG box-containing proteins (Laudet *et al.*, 1993). The Sox consensus binding motif is the heptamer 5'-(A/T)(A/T)CAA(A/T)G-3' (Harley *et al.*, 1994). Sox family members bind to the minor groove of the DNA helix, widening it and inducing a bend of 70-85°. Because of this property, they can act close to other transcription factors that bind the major groove. Therefore, they function as architectural components of chromatin apart from their roles as classical transcription factors. They are also believed to recruit to the DNA proteins that have no DNA binding activity of their own via their C-terminal transactivation domain (Ambrosetti *et al.*, 2000; Kamachi *et al.*, 1999; Kamachi *et al.*, 1998; Kuhlbrodt *et al.*, 1998; Nishimoto *et al.*, 1999; Nowling *et al.*, 2000).

Thus, Sox proteins may be able to couple extracellular signals to changes in gene expression. Recent evidence indicates that in a similar fashion to the TCF/LEF family of HMG box transcription factors, three *Xenopus* Sox genes, *XSox3, XSox17α* and *XSox17β* can bind β-catenin, the intracellular signalling component of the Wnt signalling pathway. This indicates that Sox genes could translate extracellular

signals (Wnt) to changes in transcription (Zorn *et al.*, 1999). This alleged Sox binding to β-catenin can inhibit its TCF-mediated signalling activity. This property may shared with other Sox family members that have regions of LEF homology in their C terminal (Kamachi et al., 1998; Zorn et al., 1999). However, these results have only been obtained using *Xenopus* as a model organism and high levels of of both *Sox* and *β-catenin* transcripts. No interaction has been shown at physiological expression levels, nor in other organisms (or indeed ES cells; Meng Li, unpublished).

1.4.1 Sox genes in neural development

Group B Sox family members *Sox1, Sox2* and *Sox3* have many similarities. They share extensive regions of homology even outside the HMG box, can bind the same DNA sequences *in vitro*, all have a single coding exon and share sites of expression and roles during development (Collignon et al., 1996; Kamachi et al., 1998; Wood and Episkopou, 1999). Knockout studies on *Sox2* and *Sox1* have provided some evidence for functional redundancy, with effects on homozygous null animals seen where each gene is expressed uniquely (either temporally or spatially), i.e. the epiblast at the time of implantation for *Sox2* and the lens during fibre cell elongation in *Sox1* (Nishiguchi *et al.*, 1998; Pevny *et al.*, 1998). Their expression is largely overlapping at and after the neural folds stage in mouse nervous system development, with the exception that *Sox1* is restricted to CNS progenitors whereas *Sox2* is also expressed in PNS progenitors and also in the gut endoderm.

Sox2 protein is laid down in the growing oocyte and persists throughout preimplantation development (Robin Lovell-Badge, personal communication). *Sox3* starts being expressed slightly later, and its expression is overlapping to that of *Sox2* at the epiblast component of the pre-gastrulation mouse embryo (Wood and Episkopou, 1999). They are both very early markers of neural tissue, with *Xenopus Sox2* being induced after Chordin expression (Mizuseki *et al.*, 1998), (i.e. a later onset of expression than in the mouse, restricted to neural tissue), and remain expressed in cells of the developing neural plate and tube (Rex *et al.*, 1997; Rex *et al.*, 1994; Wood and Episkopou, 1999). Mouse *Sox2* is also expressed by ES and EC cells (Yuan *et al.*, 1995). *Sox2* (as well as *Sox3*) expression is also seen in the gut (Wood and Episkopou, 1999) and the PNS (Pevny and Lovell-Badge, 1997), but not in the neural crest where PNS cells originate.

Sox1 expression in vivo comes on only at the neural folds stage, and thereafter is mostly overlapping to that of *Sox2* and *Sox3* (Wood and Episkopou, 1999). As the neural plate folds and fuses dorsally to form the neural tube, expression of all three genes is maintained. At the point of floorplate differentiation, Sox1 transcripts and protein are lost from that region, and so is Sox3 (Pevny et al., 1998; Wood and Episkopou, 1999). After mid-gestation in the mouse, expression of the three genes is maintained in the ventricular zone of the developing CNS (Wood and Episkopou, 1999). Sox1 is shown to be associated with proliferating cells of the neural tube, expression being lost concomitantly with terminal differentiation (Pevny et al., 1998). Another region of *Sox1/2/3* expression is the developing eye. *Sox1* and *2* are expressed during lens induction (Kamachi et al., 1998), and later, Sox1 is responsible for lens fiber elongation (Nishiguchi et al., 1998). *Sox2* regulatory sequences confer expression of a βgeo transgene to the neurogenic regions of adult mouse brain and neural stem cells cultured in vitro, indicating that the expression in proliferating cells of the CNS is a property it shares with *Sox1* (Zappone *et al.*, 2000).

Sox2 is known to activate the *Fgf-4* gene in combination with Oct-4, and the embryonic stem cell coactivator *Utf1* also with Oct-4 (Ambrosetti *et al.*, 1997; Nishimoto et al., 1999; Yuan et al., 1995). It appears that both Sox1 and Sox2 are mainly acting as activators in combination with other transcription factors (Nowling et al., 2000; Uchikawa *et al.*, 1999).

Known targets of Sox1 include the crystallin genes for which its function during development is critical (Kamachi et al., 1998; Nishiguchi et al., 1998). During neurogenesis the roles of Sox1 and 2 may be redundant as they can probably activate the same genes (Collignon et al., 1996). Function of Sox proteins is often dependent on specificity conferred not by the DNA binding domain, but the C terminal transactivation domain (Kamachi et al., 1999). Exchange of HMG boxes between Sox genes of different groups makes little difference to their specificity (Kamachi et al., 1999).

The pattern of expression of *Sox1/2/3* is strongly suggestive of an important role during or just after neural induction. Experiments with dominant negative Sox2 constructs injected in Xenopus embryos revealed a requirement for group B Sox transcription factors in neural induction. The first construct comprised of Sox2

lacking a DNA binding domain, while in the other the Sox2 DNA binding domain was fused to the engrailed repressor. Both constructs suppressed neural induction in animal explants and in vivo from the late blastula stage onwards (Kishi *et al.*, 2000). As group B Sox family members can bind the same sequences *in vitro* (Kamachi et al., 1999), the repressor construct could potentially work against Sox3 as well as Sox2 (no *Xenopus* Sox1 has been described).

A gain of function in vitro study had previously implicated Sox1 in neural determination. Since *Sox1* (unlike *Sox2*) is not expressed in ES and EC cells, but is rapidly upregulated during their neural differentiation, Pevny et al. (1998) misexpressed *Sox1* to see if it can drive pluripotent P19 EC cells to differentiate along the neural fate. *Sox1* overexpression could substitute for the requirement for retinoic acid in differentiation, implicating Sox1 in fate decisions during neural determination (Pevny et al., 1998).

1.5 Gene targeting

Gene targeting is the process of introducing a piece of foreign DNA into the genome at a precise location via homologous recombination. Homologous recombination in mammalian cells was first observed between homologous pieces of exogenous DNA (Folger *et al.*, 1982), but it was soon verified that such recombination can occur between exogenous DNA and chromosomal loci, albeit at a much lower frequency than had previously been described for yeast cells (Bollag *et al.*, 1989; Muller, 1999; Smithies *et al.*, 1985).

The observation that mouse embryonic stem cells can mediate homologous recombination (Thomas and Capecchi, 1987), along with their capacity to contribute to the germline of chimeras has led to the extensive use of homologous recombination to inactivate genes in the mouse genome to study loss of function mouse mutants ("knockout mice") (Capecchi, 1989). This technique has helped developmental biologists establish roles for several genes and elucidate signalling pathways and genetic interactions in mammals (Muller, 1999).

The parameters that affect the efficiency of gene targeting have been reviewed extensively since the first gene targeting experiments were reported (Deng and Capecchi, 1992; Muller, 1999). Briefly, these are the extent of homology between the targeting vector and the endogenous locus, the chromosomal locus

itself, the use of isogenic DNA in the construction of the targeting vector and (for sequence replacement vectors) the distribution of homology flanking the selectable marker.

The homologous recombination frequency increases exponentially with the length of total homology of the vector to the chromosomal locus (Deng and Capecchi, 1992), provided that both of the homology arms are longer than 1Kb (Hasty *et al.*, 1991). Isogenic DNA, derived from the same strain of mouse as the cells to be targeted gives an increased efficiency of targeting. This is because of variations in sequence (mismatches, polymorphic repeats) present in different strains that negatively affect the targeting frequency (Deng and Capecchi, 1992; Muller, 1999). In mismatch repair deficient ES cells the targeting frequency at the retinoblastoma susceptibility locus with isogenic DNA is as efficient as with non-isogenic DNA (de Wind *et al.*, 1995).

Another use of gene targeting, often used in combination with gene inactivation studies, is to identify sites of expression of the targeted gene by introduction of a histological marker. Most commonly used are the *Escherichia coli LacZ* gene encoding β-galactosidase which enables staining with the chromogenic substrate 5-bromo-4-chloro-indolyl-β-D-galactoside (X-gal), or an engineered fusion with the neomycin resistance gene (βgeo) (Friedrich and Soriano, 1991). This is possible whenever the gene targeting results in the introduction of the reporter gene under the control of endogenous regulatory and promoter sequences (Le Mouellic *et al.*, 1992; Mansour *et al.*, 1990; Mountford *et al.*, 1994).

More recently, the green fluorescent protein from *Aequorea victoria* has been employed for such studies. The advantage of GFP and its derivatives and mutants is that GFP expression can be visualised directly by fluorescent microscopy without fixation of the specimen and histological processing. This means that the expression of the targeted gene can be monitored in real time during development or the course of the experiment (Chalfie *et al.*, 1994).

1.5.1 The Cre-lox system

The Cre (causes recombination) protein is a 38-kDa recombinase isolated from the bacteriophage P1. It efficiently promotes both intra- and intermolecular synapsis and recombination of DNA both *in vitro* and *in vivo* at a specific site, called

loxP (locus of crossing over (x), P1), and does not require any other protein factors (Sternberg and Hamilton, 1981). These properties have been extensively used in gene targeting experiments initially for conditional removal of coding regions of genes upon expression of Cre (Muller, 1999). Each lox site is 34bp long and consists of two 13bp inverted repeats separated by a spacer region (Hoess *et al.*, 1982). Although the sequence of the inverted repeats is vital for Cre-mediated recombination, certain mutations in the spacer region are tolerated but alter the specificity of recombination (Hoess *et al.*, 1986). A pair of lox sites with the same mutation in the spacer region can recombine efficiently with each other, but not with the wild-type lox sites. Lox511 sites are such an example, containing a point mutation in the spacer region rendering them heterospecific to the loxP sites (Hoess et al., 1986).

1.6 Aim of the project

As described in this chapter, ES cell neural differentiation is a powerful system to study neural induction, monitor the parameters that govern lineage commitment and produce material for transplantation studies. The major limitations of this system are the disorganised mode of differentiation, qualitative interpretation of the results and the inability to study differentiation at single cell level. The aim of this project was to create a cell line that would enable the quantitative, real-time observation of ES cell specification and differentiation and the selection of pure populations of proliferative neural progenitors. The strategy employed to achieve this was the introduction of a vital reporter/selection cassette into the Sox1 locus via homologous recombination in ES cells.

2 Materials and Methods

2.1 Materials

Unless otherwise stated, all chemicals were of analytical, molecular biology or tissue culture grade, as appropriate, and supplied by BDH Laboratory Supplies or Sigma. Electrophoresis grade agarose was bought from BioWhittacker Molecular Applications (Rockland, USA). Restriction and modifying enzymes were from Roche Molecular Biochemicals or New England BioLabs and radioisotopes were supplied by Amersham Pharmacia Biotech. Synthetic oligonucleotides were synthesised by Oswel DNA service (University of Southampton, UK)

2.1.1 Solutions

1x TAE	0.04M Tris-acetate, 0.001M EDTA, pH 7.7
0.5x TBE	0.045M Tris-borate, 0.001M EDTA
20x SSC	3M NaCl, 0.3M tri-Na Citrate, pH 7.0
PBS	5 tablets of PBS salts dissolved in 1L of water
DEPC water	0.1% Diethyl pyrocarbonate in water
LB broth	1% (w/v) tryptone (Difco)
	0.5% (w/v) yeast extract (Difco)
	85mM NaCl
LB agar	1.5% (w/v) agar (Difco) in LB broth
GMEM	1x GMEM (Gibco)
	10%(v/v) foetal calf serum (Globepharm, Surrey)
	0.25% (w/v) sodium bicarbonate (Gibco)
	0.1% MEM non-essential amino acids (Gibco)
	4mM Glutamine (Gibco)
	2mM sodium pyruvate (Gibco)
	0.1mM 2-mercaptoethanol (Sigma)
	human recombinant Leukemia inhibitory factor (made in-house by D. Rout by transfection of COS-7 cells with an expression plasmid and harvesting of conditioned media) (LIF) 100U/ml
DMEM/F12	0.5x DMEM 0.5x F12(HAM) (Gibco)

N2 supplement	25μg/ml Insulin
	100μg/ml Apo-transferrin
	6ng/ml Progesterone
	16μg/ml Putrescine
	30nM Selenium Chloride
	50μg/ml BSA
N2B27 medium	0.5x DMEM/F12
	0.5x Neurobasal medium (Gibco)
	0.5x N2 supplement
	0.5x B27 (Gibco)
Trypsin solution	0.025% trypsin (Gibco)
	1.3mM EDTA
	0.1% chicken serum (Flow Labs)
	in PBS
Church & Gilbert hybridisation buffer	0.5M Na_2HPO_4 (pH 7.2)
	70g/L SDS
	1mM EDTA
Lysis buffer	10mM Tris pH8.0
	50mM EDTA
	100mM NaCl
	0.5% SDS
	0.5mg/ml Proteinase K (Roche) added just before use
SM buffer	0.1M NaCl
	10mM $MgSO_4$
	50mM Tris pH7.5
	0.01%(w/v) gelatine
Denaturing buffer	0.5M NaOH
	1.5M NaCl
Neutralising buffer	0.5M Tris pH7.5
	1.5M NaCl
Top Agar	10g/l Tryptone (Bacto)
	5g/l Yeast extract (Bacto)
	10g/l NaCl

2.2 Molecular Biology Methods

General molecular biology methods were as described in (Sambrook *et al.*, 1989). Analytical DNA electrophoresis was routinely carried out in TBE buffered agarose, whereas preparatory electrophoresis was done in TAE buffer.

2.2.1 Screening phage λ library

Phage were grown in *E. coli* LE392 cells. A single colony of bacteria was inoculated into 50ml of LB broth containing 10mM $MgSO_4$ and grown overnight at 37°C. Cells were pelleted by centrifugation at 3000g at 4°C for 15 minutes and resuspended in 10ml of 10mM $MgSO_4$. This stock of cells could be used for two weeks without any significant change in their plating efficiency.

The library screened was a 129/Ola mouse genomic DNA library in λ 2001 phage, made by Martin Kennedy, Terry Rabbitts and Andrew Smith obtained from Dr. Andrew Smith.

The library was first tested to establish the number of plaque forming units per ml of phage solution. Test plating was carried out just like the screen, only with serial dilutions of the phage. Before screening, a titration was carried out to establish exactly what volume of phage to use for optimal plaque density. This was done as for the screen, but several volumes of phage were tested and the one that gave a high number of plaques but fewest overlap between them was used to screen the library. These procedures were repeated for each fresh phage preparation.

To screen the library, 300μl of bacteria were mixed with the volume of phage established from the titration and incubated at 37° C for 20 minutes. 8ml of warm (49° C), liquid top agar were added to the mix, and immediately poured on 15cm Petri dishes of dry LB bottom agar. The dishes were swirled to evenly distribute the top agar/phage/cells mix, then incubated at 37 ° C overnight.

The next day plates were chilled at 4° C for 2h, then the plaques were filter-lifted. For the first lift, membranes (NYTRAN 0.45; Schleicher and Schuell) were placed on the plates for 45 seconds. Markings were made on the plate and membrane to help with orientation of the filter after hybridisation. Filters were then placed on Denaturing buffer for 3 minutes followed by Neutralisation buffer for 2 x 5 minutes. Each filter was then rinsed in 2x SSC and baked at 120° C for 20 minutes

between two sheets of 3MM paper. The membranes were then hybridised with the appropriate radiolabelled probe (see below). For the second lift, the same procedure was followed, except that membranes were left on the plates for 2 minutes.

Plaques that gave a positive signal after hybridisation and autoradiography on both filter lifts were picked using the wide end of a 1000μl micropipette tip and resuspended in 1ml of SM buffer containing a drop of chloroform.

Secondary and tertiary screening were done using the same procedure for each positive plaque.

2.2.2 Cloning and subcloning

Cloning of DNA fragments involved restriction enzyme digestion, gel purification and ligation. Restriction enzyme digestions were performed according to the enzyme's manufacturers instructions. Digested DNA was then ran on agarose gels (TAE) for resolution of the fragments. DNA was recovered from the gel by cutting a piece of the gel containing the desired band and extracting the DNA using Quiaquick gel extraction kit (Quiagen) according to the manufacturers instructions. The concentration of the recovered DNA was estimated by agarose electrophoresis (TBE) and a 3:1 insert:vector molarity ratio was mixed with 1U T4 ligase in 1x ligation buffer (Roche) and incubated at least 3 hours at 13°C or at 4°C overnight.

2.2.3 Transformation of bacterial cells

Competent DH10β bacteria were routinely used for transformations. Competent bacteria were prepared in the following way. A single colony from a freshly streaked plate was grown in LB broth overnight at 37°C. 1ml of the culture was used to inoculate 100ml of LB broth and was returned to a shaking incubator until it reached an $O.D._{550}$=0.3-0.6. Cells were harvested by centrifugation for 5 minutes at 3000g at 4°C. The pellet was resuspended in 5ml of TSB (10% PEG 3350, 5% DMSO, 10mM $MgCl_2$,10mM $MgSO_4$ in LB broth). The mixture was incubated on ice for 10 minutes, after which 10% glycerol was added, it was aliquoted and snap frozen in liquid nitrogen and stored at -70°C. For transformation, DNA was mixed with water to a total of 80μl. 5μl of 5x KCM buffer (0.5M KCl, 0.15M $CaCl_2$,0.25M $MgCl_2$) was added and the mix was chilled on ice. 100μl of competent cells were thawed on ice and added to the DNA mix. That was incubated on ice for

20 minutes and then at room temperature for a further 10 minutes. 800μl of LB broth were added to the cells and they were transferred to a shaking incubator at 37°C for 40-60 minutes before being plated on LB agar containing suitable selection. Recombinant bacterial colonies were screened for the presence of inserts by plasmid preparation and restriction enzyme digestion.

2.2.4 Nucleic Acid Isolation

2.2.4.1 Plasmid isolation

Plasmid purification was done from overnight cultures of single bacterial colonies in LB broth using Quiagen's minispin kit or maxiprep kit for small and large scale preparations respectively, according to the manufacturer's instructions.

2.2.4.2 Genomic DNA isolation

ES cells

ES cells grown for genomic DNA isolation were grown to overconfluence in 24-well plates or 25ml flasks. They were rinsed in PBS twice and lysed overnight at 37°C in lysis buffer. The lysate was transferred to an eppendorf and an equal volume of isopropanol was added. The tube was inverted several times until a DNA precipitate was visible. DNA was pelleted by centrifugation at 13,000rpm in a benchtop centrifuge for 10 minutes, washed in 70% ethanol and resuspended in a suitable volume of sterile water. The concentration of DNA was established by spectrophotometric analysis at 260nm.

a) Tails

Tail tips (0.5-0.7 cm) from newly weaned animals were digested overnight in lysis buffer in a 55°C shaking waterbath, and then boiled for 10 minutes. They were then centrifuged, and the supernatant was removed and mixed with an equal volume of phenol (liquefied, washed with Tris buffer, Fisher Scientific, UK). The tubes were then centrifuged for 5 minutes (13,000rpm) and the top aqueous phase containing the DNA was removed. An equal volume of a 1:1 mix of phenol:chloroform was added to that, the tube mixed and centrifuged for 5 minutes. The aqueous phase was removed and mixed with an equal volume of chloroform and centrifuged. The aqueous phase was again removed and DNA was precipitated by addition of 1/10 volume of 10M Na acetate and 2 volumes cold ethanol. The tubes were centrifuged for 10 minutes, the DNA pellet washed with

70% ethanol, dried and resuspended in 500μl TE buffer (1mM Tris-Cl, pH7.5; 1mM EDTA, pH8).

2.2.4.3 RNA isolation

Total RNA was isolated from cells using Rneasy minikit (Quiagen) according to the manufacturers instructions.

2.2.4.4 Isolation of phage DNA

10^{10} plating cells were infected with a single plaque in 10ml LB broth and incubated overnight at 37°C. This culture was used as a phage stock for isolating DNA. 2ml of cell stock were mixed with 1ml of phage stock and 200 ml LB broth containing 10mM $MgSO_4$ and were incubated overnight with vigorous shaking at 37°C. The next day, the culture was checked for lysis and 1ml of chloroform was added for 10 minutes to help complete the lysis. Bacterial debris were pelleted at 3000g for 10 minutes at room temperature. Bacterial nucleic acids present in the supernatant were digested with RNase A and DNase I (10μg/ml each final concentration) at 37°C for 1 hour. An equal volume of 20% (w/v) PEG6000, 2M NaCl was added and the mixture incubated on ice for 1 hour. Phage particles were pelleted by centrifugation at 12000g for 30 minutes at 4°C. The pellet was drained and resuspended in 10ml SM buffer and extracted with chloroform twice. DNA was extracted by adding 0.5ml 0.5M EDTA (pH8), 1ml 5M NaCl and mixing with TE buffered phenol followed by centrifugation for 10 minutes. Phenol extraction was repeated and the aqueous phase was extracted with chloroform. DNA was precipitated with 2 volumes of ethanol, washed in 70% ethanol and air-dried before resuspending in TE.

2.2.5 Southern blotting

10μg of genomic DNA were digested with 80U of restriction enzyme for 3 hours in the appropriate buffer and temperature, and then another 40U were added for another 1-2 hours. The digested DNA was ran on a 0.8%-1.2% agarose gel (TAE) overnight. The next morning, the gel was photographed under short wave UV light to nick the DNA, and then placed in denaturing buffer for 20 minutes with light agitation. The buffer was replaced and incubation repeated, and then the gel was

soaked in neutralising buffer for 2x 20 minutes. Then the gel was blotted onto a nylon membrane in 20x SSC overnight. Following transfer, the membrane was washed in 2x SSC and then baked at 120°C for 20 minutes. The membrane was then hybridised with the appropriate radiolabelled probe

2.2.6 Radiolabelling of DNA

DNA was labelled with HighPrime labelling mix following the manufacturers instructions. Briefly, 20-40 ng of linear, denatured DNA template were incubated with HighPrime (containing buffer, nucleotides, random hexamers and polymerase) and 40μCi ^{32}P-dCTP for 20-40 minutes at 37°C. Unincorporated nucleotides were removed by passing the reaction mixture through a ProbeQuant G50 spin column (Amersham Pharmacia Biotech). The reaction product was then denatured by boiling for 5 minutes, placed on ice for 2-3 minutes and added to the hybridisation mixture. Routinely, the probes measured 1.5x10^6 cpm/ml of radioactivity.

2.2.7 Hybridisation of membranes with radioactive probes

Membranes were pre-hybridised for 1 hour in ~20ml Church & Gilbert buffer (Sambrook et al., 1989) at 65°C. After the addition of probe, membranes were hybridised overnight at the same temperature. The next day, the hybridisation mix was removed and the membranes washed in 2x SSC, 0.1% SDS twice for 20 minutes followed by two washes in 0.2x SSC 0.1% SDS, before light air-drying. They were subsequently wrapped in Saran wrap and exposed to either film (KODAK X-omat) in an intensifying screen at -70°C for 1-7 days or in a phosphorimager screen for 1 hour-2days.

2.2.8 Polymerase chain reaction (PCR)

The PCR was performed in a GeneAmp 9700 thermal cycler from Perkin-Elmer Applied Biosystems.

a) Fusion of *egfp* to *Sox1*

100μg of plasmid pCAGGFP IP was amplified using *Pfu* polymerase (Promega). The reaction also contained, in 50μl, 5μl of 10x buffer, 7.5μl and 6μl of primers MpsFwd3 and MpsRVLS3 respectively (0.1μg), 1μl (25μM) dNTP and 1μl *Taq* polymerase. The amplification conditions were:

94°C 5 minutes
94°C 30 seconds
58°C 45 seconds
72°C 2 minutes
72°C 45 seconds
} 30 cycles
72°C 10 minutes
4°C

c) Fusion of *neo* to *Sox1*

170μg of plasmid pMGD20neo was amplified using *Pfu* polymerase (Promega). The 50μl reaction also contained 5μl of 10x buffer, 7.5μl and 4μl respectively of primers MPSFWDneo1.1 and MPSRVSneo1 (0.1μg), 1μl (25μM) dNTP and 1μl *Pfu* polymerase. The amplification conditions were:

94°C 5 minutes
94°C 30 seconds
60°C 45 seconds
72°C 45 seconds
72°C 45 seconds
} 20 cycles
72°C 10 minutes
4°C

Following PCR amplification, 1μl of *Taq* polymerase was added to the reaction and the tube incubated at 72°C for 10 minutes to enable TA cloning of the product.

2.2.9 DNA sequencing

Was carried out by D. Kivlichan using the BigDye dideoxy termination sequencing kit and a Perkin Elmer ABI-Prism automated DNA sequencer.

2.3 Cell culture methods

All cells were maintained at 7.5% CO_2 at 37°C in a humidified incubator (Heraeus, B5060 EC/CO2). All manipulations were undertaken in a laminar flow sterile hood (ICN). To avoid bacterial or fungal contamination of the cultures, all objects and surfaces were wiped with 70% industrial methylated spirits (IMS) before commencing work. Tissue culture grade plastics were supplied by Iwaki and Nunc.

2.3.1 Routine culture of ES cells

ES cells were cultured on 0.1% gelatine-coated plastic in GMEM supplemented with human recombinant leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988) as described previously (Smith, 1992). LIF was prepared by D. Rout by transfecting COS-7 cells with a human LIF expression plasmid and harvesting the supernatant. The latter was tested on ES cells by limiting dilution, and the smallest amount capable of maintaining self-renewing colonies was designated as one unit per ml . Routinely, 100 units of LIF per ml were used on the cultures.

To passage the cells, the medium was removed and the cells rinsed twice with PBS. Trypsin solution was added to just cover the cells and the flask was incubated at 37°C for 2-3 minutes. The trypsin activity was quenched by addition of 10ml of medium, and cells were pipetted into a single cell suspension. The cell suspension was then centrifuged (200g, 3min.) and the cell pellet resuspended in 10 ml of warm medium. An aliquot of the cells was seeded into a new flask containing pre-warmed medium. Routinely, cells were passaged at a 1:5 –1:10 split every 2-3 days.

2.3.2 Freezing and thawing cells

Cells in flasks were trypsinised to a single cell suspension and centrifuged at 200g for 3 minutes. The cell pellet was resuspended in 1ml of freezing mix (medium containing 10% dimethyl sulfoxide, AnalaR, BDH) and transferred into a cryotube (Nunc). The latter was placed in -80°C overnight and then was transferred to a liquid nitrogen cell bank (XCL110, Minnesota Valley Engineering Cryogenics).

To freeze cells in plates, the medium was removed and freezing mix was added just to cover the cells. The plate was then transferred to a polystyrene box and stored at -80°C (Ure *et al.*, 1992).

To thaw cells frozen in vials, the vial was removed from the liquid nitrogen bank and thawed in a 37°C waterbath. The contents were transferred into a 30ml Universal tube containing 10 ml of pre-warmed medium and centrifuged. The cell pellet was gently resuspended in 10 ml of medium and seeded into a 25cm^3 flask.

To thaw cells frozen in plates, 1ml of pre-warmed medium was added into each well. The plate was then held between hands until thawed. Two-thirds of the medium were carefully aspirated and replaced with fresh medium (Ure et al., 1992).

2.3.3 Culture of COS-7 and 10T1/2 cells

These cells were grown on tissue culture plastic in medium without LIF. Passaging, freezing and thawing were as described for ES cells.

2.3.4 Differentiation of ES cells

Neural differentiation of ES cells was performed in 6cm tissue culture dishes (Nunc) or 6-well plates (Iwaki) coated with 0.1% gelatin. Routinely, $2x10^5$ cells were plated onto each plate in N2B27 medium. Medium was changed every two days. To quantify the differentiation, cells were dissociated using 0.5 ml of trypsin solution, then trypsin was quenched with 0.5ml of PBS containing 10% (v:v) fetal calf serum. The number of cells was counted, and then the cells were analysed in a flow cytometer. When exogenous effectors were used during the differentiation, these were added from the time of plating and changed with the medium every two days. Effectors added were: recombinant human BMP-4, recombinant mouse Chordin and recombinant mouse Noggin/Fc chimera (R&D Systems, reconstituted in PBS+1%BSA and used in dilutions 1:1000-1:500), SU5402 (Calbiochem, reconstituted in DMSO and used diluted 1:5000 or less), PD98059 (New England Biolabs and Promega, diluted in DMSO and used at 1:10000).

For embryoid body differentiation, cells were plated onto bacterial grade Petri dishes in GMEM and allowed to form embryoid bodies. Medium was changed every two days. On day 4, 10^{-6}M all-trans retinoic acid was added and the embryoid bodies cultured for a further 4 days in GMEM + retinoic acid. On day 8, embryoid bodies were washed with PBS, trypsinised with 4x trypsin for 5 minutes and dissociated into single cells before plating or FACS analysis.

2.4 Introduction of DNA in cells

2.4.1 Lipofection of COS-7 and 10T1/2 cells

$2x10^5$ cells were plated on each well of a 6-well plate the day before the transfection. The next day, 95μl of serum free GMEM (prepared as normal but

without serum) were mixed with 5μl of FuGene transfection reagent (Roche)and left at room temperature for 5 minutes. After that time, the mix was added onto a drop of 2μg of DNA and left at room temperature for 15 minutes. It was then added to the cells which were returned to the incubator and left overnight. Cells were photographed the next day.

2.4.2 Electroporation of ES cells

Confluent flasks of E14Tg2a ES cells (passage 15) were trypsinised and 10^7 cells pelleted and resuspended in 0.7ml of PBS. 150μg of *SalI* linearised targeting vector DNA resuspended in 0.1ml of PBS were added to the cells, and the cell-DNA mix was transferred to a sterile electroporation cuvette (BioRad 0.4cm gap) for electroporation. This was performed in a BioRad Genepulser electroporator at 0.8kV and 3μF. Immediately after the electroporation, the cells were plated onto 10cm gelatinised tissue culture plates at a density of 10^6 cells per plate. 24 hours after the transfection, the medium was changed to selection medium containing 100-200 μg/ml hygromycin B (Roche). Cells were grown in selection medium for 7-10 days until colonies appeared. Single colonies were picked into 24-well plates coated with gelatine. Cells were expanded and each clone was split into two plates, one of which was frozen as a stock (Ure et al., 1992), while the other one was used for DNA extraction and Southern blot analysis.

For the transient transfection with Cre, cells were grown in a medium flask ($75cm^3$), tryspinised and resuspended in 0.7ml PBS. They were then mixed with 50μg of plasmid pCAGGSCreIP (which contains the gene encoding for Cre recombinase under the control of the CAG promoter (Niwa *et al.*, 1991) linked to the *pac* gene conferring puromycin resistance) and electroporated as normal. The cell suspension was then transferred to a fresh medium flask and grown for 2 days without any selection, to enable efficient excision of the lox511 flanked cassette and loss of the plasmid without it integrating. After two days, the cells were trypsinised, counted and 10^2-10^4 cells were plated on 10cm plates. Ganciclovir was added to the plates 2 days later and resistant colonies appeared after 5-7 days. Colonies were picked, expanded and screened as for the targeting experiments.

2.5 Histological analysis

2.5.1 Immunocytochemistry

For immunocytochemistry, cells were rinsed twice in PBS and fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. They were rinsed in PBST (PBS with 1% Tween20) twice for 5 minutes to permeabilise the cell membrane, and then incubated in PBST with 3% serum and 10% bovine serum albumin (BSA) (blocking solution) to prevent non-specific binding of the antibody. They were rinsed twice in PBST again, and incubated in the primary antibody at the appropriate dilution in blocking solution either at room temperature for 2 hours or overnight at 4°C. After that incubation, cells were rinsed 3 times with PBST and incubated for 2-3 hours with the secondary antibody in blocking solution in the dark. After that time, the secondary antibody was removed and the cells washed 3 times with PBS before observing under a microscope for staining.

2.5.2 Image acquisition and manipulation

Images were captured via a Zeiss Axiovert inverted microscope (or a Nikon SMZ-U dissection microscope for whole embryos) using a Hamamatsu Orka colour camera and Improvision Openlab 2.2.5 software on a Macintosh G3 computer. Binning was used for weak fluorescent samples and pseudocolour was applied with Photoshop software. Contrast and brightness enhancement of acquired images was also performed with Photoshop to enhance weak fluorescence of some images and improve the contrast.

2.5.3 Cryosections

For cryostat sections of animal tissue, dissections were carried out in PBS and then the embryo or tissue was cryopreserved in PBS containing 30% sucrose overnight before being embedded in O.C.T. compound (BDH) and frozen on a bed of dry ice. For sectioning, the frozen block was equilibrated to the appropriate temperature (generally about -18°C) in the cryostat chamber. Frozen sections were taken at 20μm using an AS620 Cryotome (Anglia Scientific Instruments), thaw mounted onto polysine coated microscope slides (BDH) and observed on a microscope.

2.6 Animals

Mice were housed and bred within the University of Edinburgh, according to the Animals (Scientific Procedures) Act (UK)1986. They were maintained on a cycle of 12 hours dark:12 hours light with the midpoint of the dark cycle at 1am. Litters from natural matings were left with parents until 3 weeks of age when they were weaned by separating the offspring from the parents. At weaning the animals were sexed and tail tips taken for genotyping. At 6 weeks of age the animals could be used for mating. Breeding setup, tail biopsies and maintenance of the animal stocks was performed by the Biomedical Unit staff in the Centre for Genome Research.

2.7 Blastocyst injection and chimera production

Chimeric mice were produced based on protocols previously described (Bradley et al., 1984; Robertson, 1987b). All the blastocyst injections and transfers were carried out by J. Nichols or S. White. Briefly, Recombinant ES cell clones were injected into C57BL/6 blastocysts. Blastocysts were usually cultured for 3 hours to allow the blastocoel to fully expand prior to injection. ES cells were trypsinised, washed once and 15-20 cells injected into each blastocyst. Around 10 injected blastocysts were transferred to the uterine horn of a pseudopregnant recipient mouse. Chimeric pups were identified by coat colour chimerism. E14 ES cells contain the A^w gene (white bellied agouti) and $c^{ch}c^{ch}$ for chinchilla at the albino locus. C57BL/6 mice are nonagouti (a) resulting in black colour. Depending on the origin of the hair follicle and the hair, the coat of chimeric mice can have black, agouti, cream or cream agouti colour.

2.8 Flow cytometry and sorting

For flow cytometric analysis, cells were trypsinised and resuspended in PBS with 10% FCS (FACS wash). Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. 10,000 events were scanned. Cell debris and some dead cells were excluded from the analysis based on electronic gates set using forward scatter (size) and side scatter (cell complexity) criteria. For timecourse experiments, the settings were determined before the experiment commenced using appropriate positive and negative controls, then saved and used for all of the time points of the experiment to ensure consistency of data acquired.

For sorting, cell suspensions in FACS wash were kept on ice and appropriate numbers of selected populations were sorted either directly into multiwells or into tubes containing N2B27 medium. To eliminate contamination, after sorting cells were grown in the presence of penicillin/streptomycin (Gibco). The sorting was done on a Cytomation MoFlo flow cytometer operated by Steve le Moenic.

3 Targeting *Sox1* in Embryonic Stem Cells

3.1 Introduction

In order to investigate further the population of *Sox1* expressing cells after ES cell differentiation and *in vivo* in developing embryos, we employed the use of gene targeting. In this Chapter, the isolation and mapping of the *Sox1* genomic locus, the rationale of the targeting vector, its construction, the gene targeting experiment and the analysis of homologous recombinants will be presented.

As described in chapter 1, the use of a vital marker was deemed necessary for the project, so the *Aequoria victoria* green fluorescent protein was employed as a reporter. GFP has been extensively studied and several mutants have been made including some with enhanced fluorescence, optimised codon usage for mammalian expression and even altered fluorescence wavelength. One such commonly used mutant is EGFP which differs from wild-type GFP in two amino acid substitutions at positions 64 and 65 (Phe-64 to Leu, Ser-65 to Thr) and in the codon usage which has been optimised for mammalian expression. The amino acid substitutions render EGFP 35 times more fluorescent than wild-type GFP when excited at 488nm, the wavelength most commonly used for excitation in flow cytometry (Cormack *et al.*, 1996). This variant of GFP was used for the construction of the targeting vector.

3.2 Library screen and subcloning

The aim of the targeting was to introduce the dual reporter/selection cassette *egfp*-IRES-*pac* into the endogenous *Sox1* locus. This cassette consists of the gene encoding for enhanced green fluorescent protein (*egfp*) linked via a mouse encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) (Mountford et al., 1994) to the gene encoding for puromycin N-acetyltransferase (*pac*). Upon transcription, this cassette will create a bicistronic transcript which will be translated to make two proteins; eGFP and puromycin N-acetyltransferase, conferring cell autonomous green fluorescence and resistance to the drug puromycin (Niwa *et al.*, in preparation).

The *Sox1* gene (as well as most *Sox* genes) has a monoexonic structure (Malas *et al.*, 1997; Malas *et al.*, 1996; Pevny and Lovell-Badge, 1997). A phage lambda2001 genomic DNA library of 129Ola strain mouse was screened with a

2046bp *EcoRI-XhoI* fragment of plasmid pmSox-1 cDNA (7a) (a gift from Dr. L.H. Pevny, Sheffield University) containing all of the *Sox1* open reading frame. The particular strain was selected because it is isogenic to the ES cells to be targeted, i.e. the ES cells are derived from that strain (129/Ola). Use of isogenic DNA for a targeting vector is established to give better targeting efficiency than use of non-isogenic DNA (Deng and Capecchi, 1992). Initially, 1.2×10^8 plaques were screened and 7 gave a positive signal. These were screened again to eliminate mixed clones. In this second round, 3 clones hybridised with the *Sox1* probe, the process was repeated for them. Finally, 2 clones (a and b, Figure 3.1A) were found positive and phage DNA was isolated for further analysis.

Initial restriction mapping identified unique restriction endonuclease sites and Southern hybridisation was performed with a Sox1 cDNA probe to verify the identity of the clones. The two clones were then restriction mapped with a variety of enzymes and then clone *a* was subcloned as three *XhoI-EcoRI* fragments into pBluescript II SK+(pBS) (Figure 3.1). The most 5' part of the clone, from the polylinker *XhoI* to the first *EcoRI* site (2 Kb) was cloned into the *XhoI-EcoRI* sites of pBS to make pSox1pr1. The 7.1 Kb fragment from that *EcoRI* site to the *XhoI* site in the *Sox1* 3' untranslated region (UTR) was cloned in pBS to make p7.1 5'Sox1, and the third fragment (4.5 Kb) of clone a, from the *XhoI* in the 3' UTR until the last *EcoRI* to make p4.51 3'Sox1 (Figure 3.1B).

These three plasmids were sequenced using the T3 and T7 primers. The end of the sequence obtained with the T3 primer on p7.1 5'Sox1 (from the *XhoI* end of the insert) was shown by homology search (using BLAST) to be part of the *Sox1* gene (Kamachi *et al.*, 1995). This verified that this clone was indeed *Sox1* .

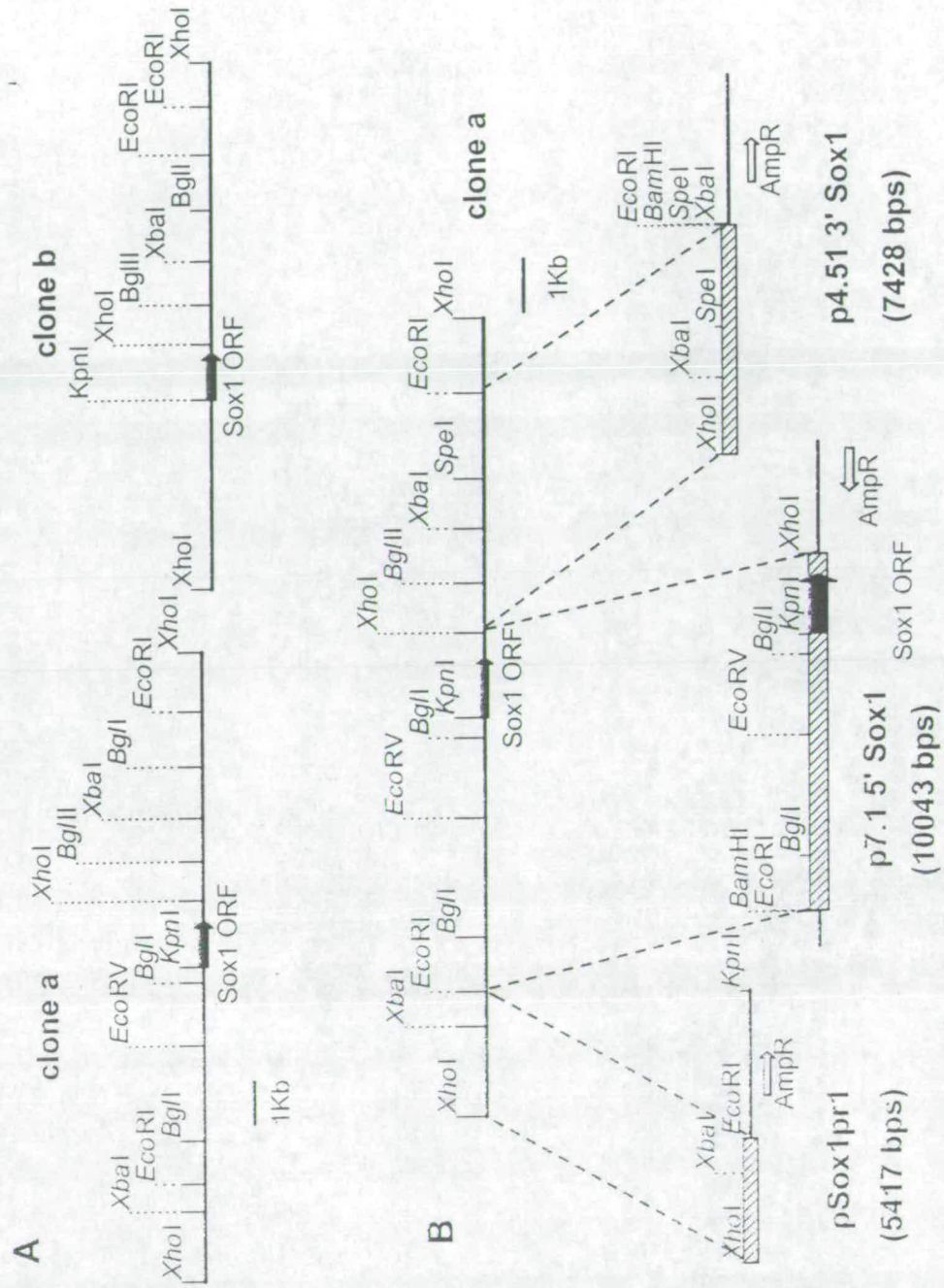


Figure 3.1 Genomic DNA phage clones

A: Partial restriction map of inserts of phage clones a and b. The extreme *XhoI* sites are of the phage polylinker B: Partial restriction maps of plasmids containing subcloned fragments of clone a. Corresponding positions to the original λ clone are indicated.

3.3 Fusion of *egfp* to *Sox1* untranslated sequences

To ensure faithful expression of the eGFP transgene from the endogenous *Sox1* promoter, it was decided to fuse *Sox1* to *egfp. Sox1* contains 4 in-frame ATG codons at its 5' end that could potentially initiate translation. However, it has been proposed (Kamachi et al., 1998) that it is not the first ATG codon that initiates translation of SOX1, because it is a poor fit to the Kozak consensus sequence for translation initiation (Kozak, 1989), but the second, which is a better match. Therefore, it was decided to fuse eGFP to the second in frame ATG of *Sox1*. To do this, PCR primers mpsFwd3 and mpsRLVS3 were designed to amplify the entire *egfp* ORF from pCAGGSeGFP IP which contains the *egfp* IRES *pac* cassette under the control of the CAG promoter (CMV immediate-early enhancer, chicken β-actin promoter and first intron, β-globin splice acceptor (Niwa et al., 1991)), using a 5' primer that would introduce a fusion to the *Sox1* leader sequence and first few codons (Table 3.1, Figure 3.2). The resulting fusion construct has the 5' UTR (up to the *KpnI* site 37 nucleotides upstream of the first ATG) and possible first three codons of *Sox1,* with the first codon of *egfp* replacing the second in frame *Sox1* ATG.

The encoded protein, depending on which ATG actually initiates translation, would be either a fusion of three amino acids of Sox1 on the N terminus of eGFP or eGFP alone. This PCR product was cloned using TA cloning in pCR2.1 TOPO (Invitrogen), and sequenced fully using M13 reverse and T7 primers. The sequence was verified by homology search to contain no mutations introduced by PCR or otherwise. The cloned PCR product was excised as an *EcoRI* fragment and cloned into *EcoRI* -digested pCAGGSeGFPIP replacing the wild-type *egfp* present there to make pSox1EGFPIPpA. To further verify that the cassette carried no mutations and that the fusion did not noticeably affect the level of eGFP fluorescence, this plasmid was transiently transfected into COS-7 and $10T^1/_2$ cells by lipofection. The cells transfected with the fusion plasmid were as bright as those transfected with the parental plasmid pCAGGSeGFPIP (Figure 3.3). The expression level however was probably higher than it would be in the targeted locus, because of multiple copies of the vector and the presence of a strong promoter.

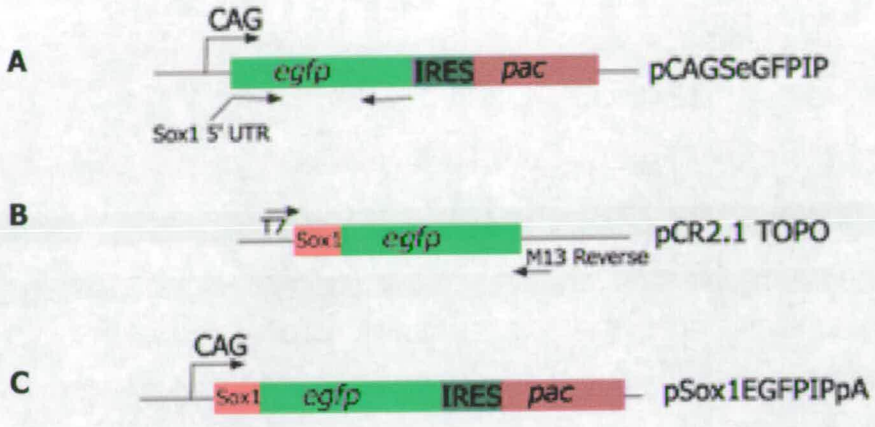


Figure 3.2 PCR fusion strategy

A Strategy for introducing the 5' fusion to *egfp* by PCR (primers indicated) B The fusion product shown schematically in pCR2.1 TOPO (T7 and M13 reverse primer sites used for sequencing are indicated) C The fusion product in the final plasmid pSox1EGFPIPpA

3.4 Vector construction

Plasmids p7.1 5'Sox1 and p4.51 3'Sox1 were further mapped by restriction enzyme digestion (Figure 3.1B) to identify useful restriction endonuclease recognition sites for the assembly of the targeting vector. It has been shown (Deng and Capecchi, 1992; Thomas and Capecchi, 1987) that the targeting efficiency is exponentially dependent on the extent of homology to the target, so the targeting vector was designed to incorporate the longest practical homology to the *Sox1* genomic locus, given the restriction map information and genomic clones available. We aimed to replace the entire *Sox1* ORF with the reporter/selection cassette eGFP IRES *pac*, so that it would be under the transcriptional control of the endogenous *Sox1* regulatory sequences, with minimum alteration to the non coding sequences.

Therefore, the use of a sequence replacement vector was deemed necessary (Thomas and Capecchi, 1987). Homologous integration of the targeting vector results in the replacement of 1587bp of *Sox1* sequences (all of the ORF and 412 bp

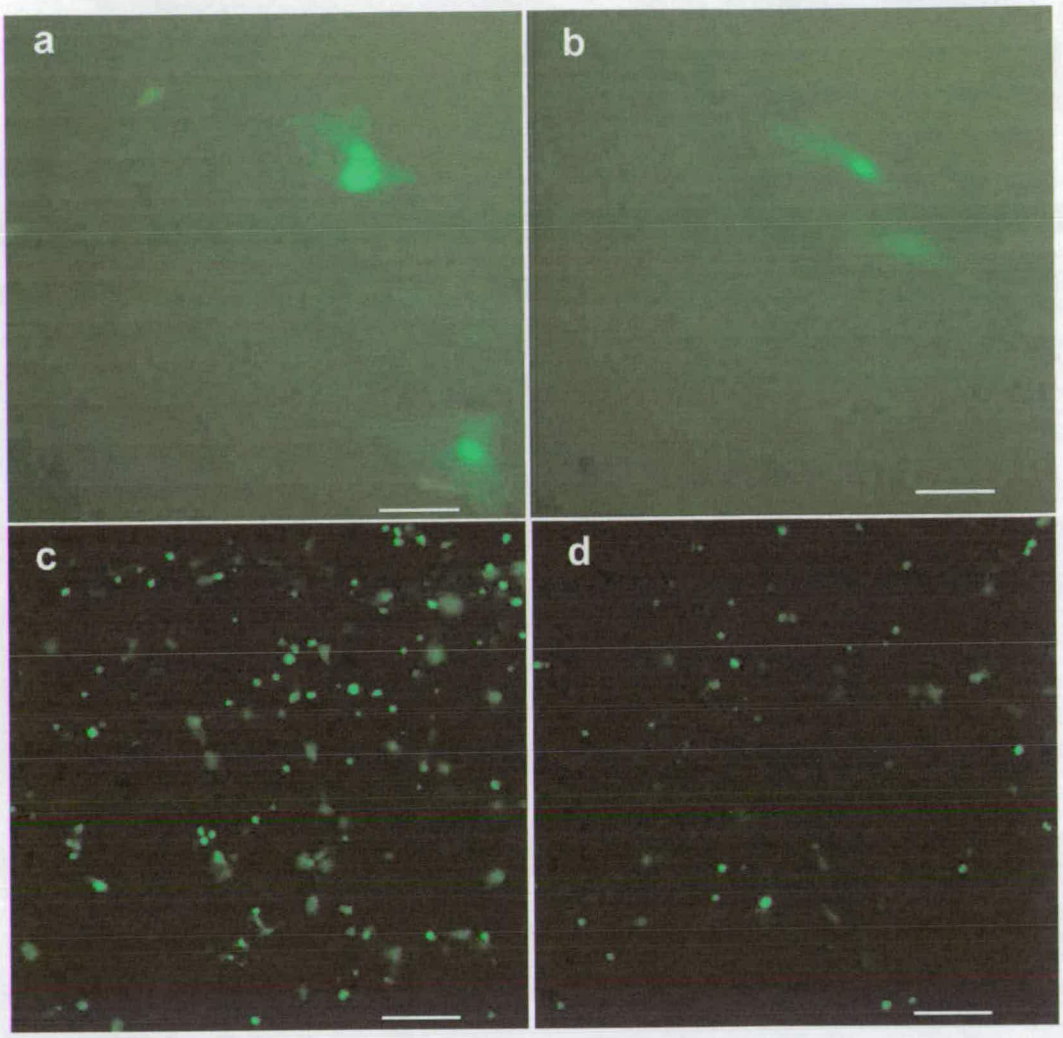


Figure 3.3 Transient transfections

a-b: 10T½ cells transfected with plasmids pCAGegfpIP and pCAGSoxegfpIP respectively. Scale bar =50μm. c-d: COS-7 cells transfected with the egfp and Sox-egfp constructs respectively. Scale bar =100μm.

of the 3' UTR) with the reporter/selection cassette. The length of total homology was decided to be about 8 Kb. This length of homology is considered sufficient for a targeting vector to target efficiently (Deng and Capecchi, 1992), and is short enough in order to be cloned easily and to be introduced efficiently into ES cells. The restriction sites that were suitable for the vector construction and screening strategy dictated that the 5' homology arm would be a 5.5 Kb *EcoRI-KpnI* fragment from p7.1 5'Sox1, and the 3' homology arm would be a 2.5 Kb *XhoI-SpeI* fragment from p4.51 3'Sox1. The vector in which the construct was put together was pUC19polymps (pUC19 with a modified polylinker). To modify the pUC19 polylinker, a set of two complementary oligonucleotides (Link1.1 and Link1.2) that contained the required restriction endonuclease recognition sites was ordered, annealed and cloned into the *XbaI-HindIII* sites of pUC19 to create pUC19polymps.

The sequence of Link1.1 and Link1.2 was such, that it replaced all the restriction sites between *XbaI* and *HindIII* on the pUC19 polylinker with *XhoI*, *SpeI* and *SalI* (table 3.1). To construct the targeting vector, the 5' homology (5.5 Kb *EcoRI-KpnI* fragment) was cloned from p7.1 5'Sox1 into pUC19polymps to give pSox1KO1. Then, the 3' homology arm (2.5 Kb *XhoI-SpeI* fragment) from p4.51 3'Sox1 was cloned into pSox1KO1 to generate pSox1KO2.
A 873 bp *KpnI-EcoRI* fragment from pSox1eGFPIPpA containing the fusion product (see section 3.3) was three-way cloned with a *EcoRI-BamHI* fragment from pCAGGSeGFPIP containing IRES-pac polyA into the *KpnI-BamHI* sites of pSox1KO2 to generate pSox1KO3.

The final step in the construction of the vector was to insert a selectable cassette for selection in ES cells. To this end, plasmid pCMVHyTk was used as a basis. This plasmid encodes for the fusion protein HyTK which results from fusing the hyrgomycin resistance gene *hph* with the HSV *Thymidine Kinase* (*TK*) gene (Lupton *et al.*, 1991). This is under the control of the CMV Immediate Early Enhancer (CMV IEE) which gives high expression in ES cells. This plasmid was modified in the following way: A *SalI* site present in the linker between *HyTK* and the polyadenylation signal was destroyed by digestion, end fill and religation. The same technique was used to eliminate a *BglII* site in the same linker. The resulting plasmid, pCMVHy-TK.1-Bgl, was digested with *XhoI* and *BamHI* and the fragment

containing the CMV*HyTK* cassette was cloned into the *SalI-BamHI* sites of pSP72poly1 to make pCMVinPSP. This was in turn digested with *BamHI* and the fragment with the cassette was cloned into the *BglII* site of pBSlox2 to create pCMVinlox. In this plasmid, the CMV*HyTK* cassette is flanked by lox511 sites in front to back orientation. A *BamHI* fragment containing the lox511 sites and the cassette within them was then cloned into pSox1KO3 to make pSox1KO4, the final targeting vector. The orientation of the CMV*HyTK* cassette was chosen to be the same as that of the eGFPIP cassette (Figure 3.4).

Table 3.1

The sequence of the oligonucleotides used in the construction of the targeting vector. Restriction sites are indicated: *XbaI*, *XhoI*, *SpeI*, *HindIII*, *KpnI*. For the fusion primers, *Sox1* sequences are shown in italics. The two in-frame ATGs of *Sox1* are shown in bold.

Link1.1	5′ CTAGACTCGAGCTAGCACTAGTGTCGAC 3′
Link1.2	5′ AGCTGTCGACACTAGTGCTAGCTCGAGT 3′
Annealed Link1.1 &1.2	CTAGACTCGAGCTAGCACTAGTGTCGAC TGAGCTCGATCGTGATCACAGCTGTCGA
MpsFwd3	5′ *GGAATTCGCGGTACCGGTGAACCCGCTAGCCGCCC AG**ATG**TACAGC**ATG*** GTG AGC AAG GGC GAG 3′
MpsRLVS3	5′ AGC CAC CAC CTT CTG ATA GG 3′
MPSFWDneo1.1	*5′CGGTACCGGTGAACCCGCTAGCCGCCCAG**ATG**TAC AGC**ATG*** GGA TCG GCC ATT GAA CA 3′
MPSRVSneo1	5′ TGC TTC CGG CTC GTA TGT TG 3′

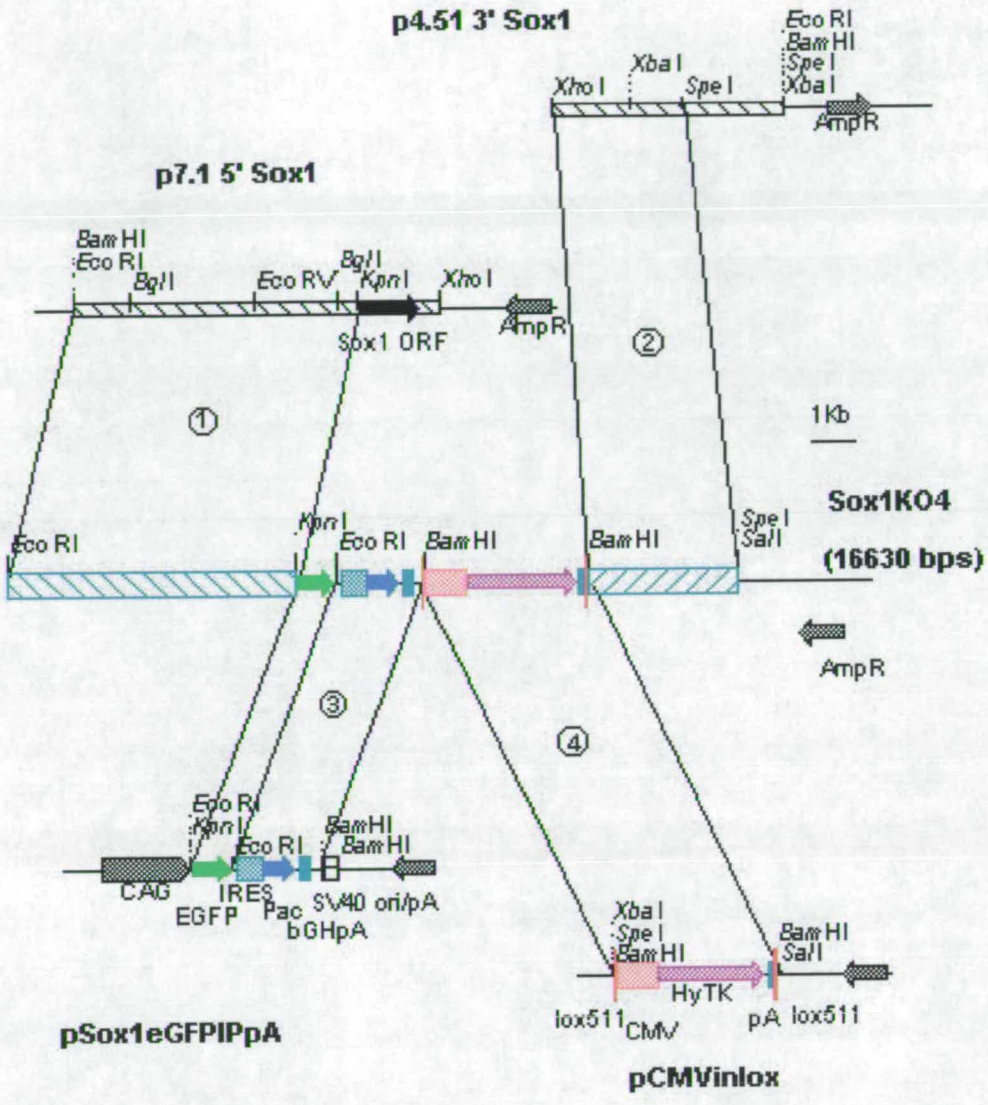


Figure 3.4 Targeting vector construction

Diagram of the four stages of the construction of the targeting vector pSox1KO4.

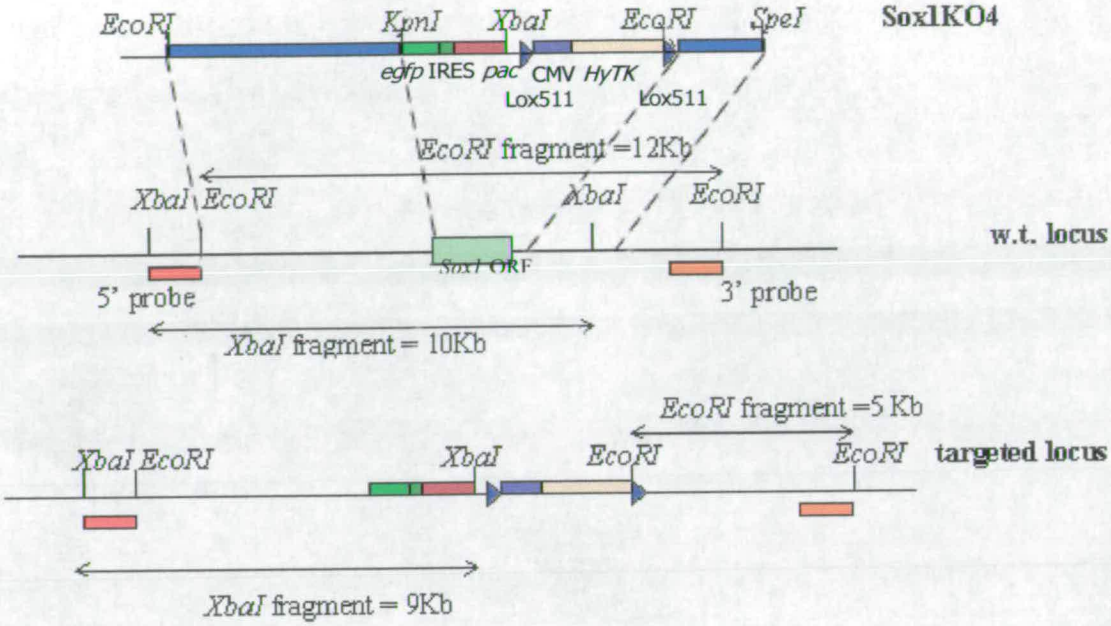
3.5 Screening strategy for homologous recombinants

To screen for correctly targeted clones, DNA was screened by Southern hybridisation with probes flanking the integration site in the *Sox1* locus. The 5' flanking probe was a ~1Kb *XbaI-EcoRI* fragment from plasmid pSox1pr1, immediately upstream of the 5' homology arm. Wild-type genomic DNA cut with *XbaI* and hybridised with this probe gives a 10Kb band, which is reduced to 9Kb upon correct integration of the targeting vector due to the presence of a *XbaI* site in the targeting vector. The 3' flanking probe was a 1.5Kb *EcoRI-BglI* fragment from plasmid p4.51 3'Sox1. This gives a 12Kb wild type band on *EcoRI* digested genomic DNA which is reduced to 5Kb upon correct integration (Figure 3.5).

3.6 Targeting Sox1KO4 in ES cells

The targeting vector was introduced into E14Tg2a ES cells (Hooper *et al.*, 1987) as described in Chapter 2. Cells were electroporated with plasmid pSox1KO4 linearised with *SalI*, selected for 6-8 days in 100μg/ml hygromycin C, colonies picked, expanded and genomic DNA was prepared from each clone.

From the 200 clones picked, 100 were screened with both probes and 3 showed correct targeting at both ends (clones 14, 46 and 53, Figure 3.8). The clones were also screened with an *egfp* probe on *XbaI* digested DNA to verify that there was only one cassette integrated into the genome. Two out of three clones gave a single 9Kb band (Figure 3.8) which indicates that they only contain one copy of the targeting vector. Clone 14 contained multiple copies and was excluded from any further analysis. The targeting frequency is therefore 3% in this single experiment.



Strategy for screening for correctly targeted ES cells by Southern blotting

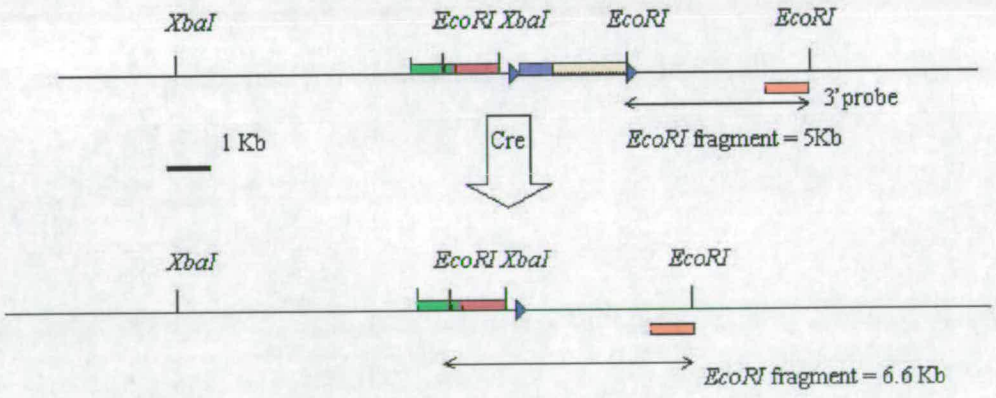


Figure 3.6 Screen for correct excision

Cre- mediated deletion of the CMV-HyTK cassette at the lox511 sites causes increase in the size of the *EcoRI* fragment hybridising to the 3' probe

3.7 Excision of selection cassette

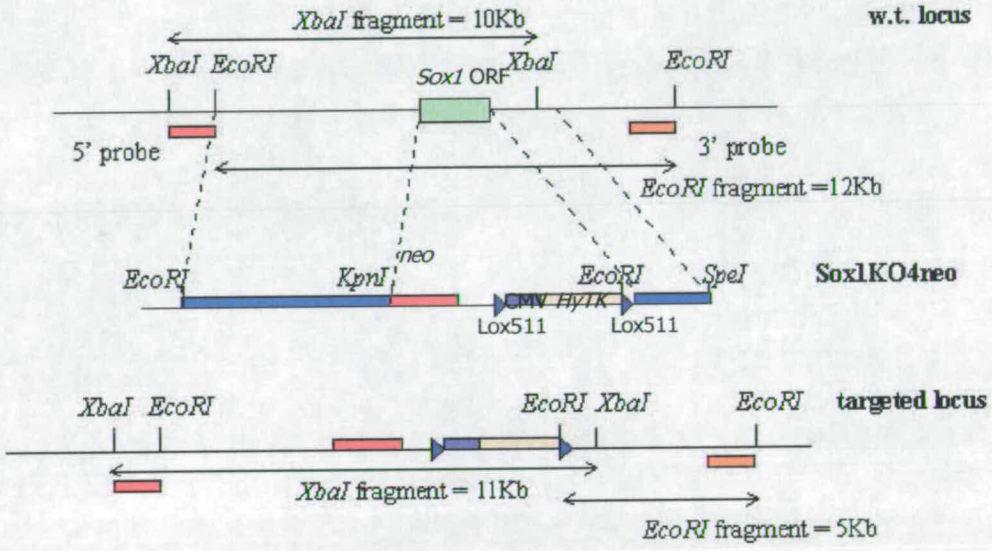
It is known (Al-Shawi *et al.*, 1988; al-Shawi *et al.*, 1991; Braun *et al.*, 1990; Ellison *et al.*, 2000) that male transgenic mice carrying the TK gene rarely transmit it to their offspring. In order therefore to get ES cells able to transmit the Sox1-eGFP reporter construct through the germline, the selection cassette CMV*HyTK* was removed from correctly targeted clones.

This was achieved by Cre- mediated site specific recombination at the lox511 sites flanking the CMV*HyTK* cassette. Clones 46 and 53 were transiently transfected with plasmid pCAGGSCreIP in which Cre recombinase is under the transcriptional control of the CAG promoter (CMV IEE, chicken β-actin promoter, β-globin intron). After negative selection with ganciclovir, colonies were picked and screened with the 3' probe for excision of the CMV*HyTK* cassette (Figure 3.6). One correctly excised clone was obtained from clone 46 (46C, Figure 3.10). The experiment was later repeated and more correctly excised clones obtained (not shown).

3.8 Second vector

Very little is known about the level of *Sox1* expression during ES cell differentiation. It has been proposed that, to get detectable fluorescence from eGFP in cells, around 10,000 molecules of the eGFP protein need to be present in the cytoplasm (Patterson *et al.*, 1997). Furthermore, puromycin resistance depends on a high expression of the *pac* gene, and there is evidence (Borman *et al.*, 1995; Martinez-Salas, 1999) that IRES mediated translation is less efficient than cap regulated translation. It was therefore conceivable that the levels of *Sox1* expression would not be sufficient to produce high enough levels of protein in order to utilise either egfp to visualise, or puromycin resistance to select for, *Sox1* expressing cells. To circumvent this possible problem, a second vector was designed and constructed to target *Sox1* with *neo*, the gene encoding for the resistance of cells to the drug G418. *Neo* has been widely used as a selectable marker in gene targeting, and gives resistance even in cases of low expression (for example, the LIFR gene; (Chambers *et al.*, 1997; Li *et al.*, 1995)) (Hanson and Sedivy, 1995). This vector was very similar to pSox1KO4 in that it utilised the same homology and the same selection cassette for selection in ES cells (since *Sox1* is not expressed in

ES cells). A PCR strategy was also used to fuse the *neo* coding sequence to the *Sox1* 5'-UTR (see table 3.1 for primer sequences of primers MPSFWDneo1.1 and MPSRVSneo1), and the resulting product was TA cloned in pCR TOPO2.1. After sequencing to verify that no mutations were introduced by PCR, a *KpnI-BamHI* fragment containing the *Sox1-neo* fusion was cloned in pSox1KO2 to make pSox1KO3neo. The final step was to clone the *BamHI* fragment containing the CMV-HyTK cassette between lox511 sites into pSox1KO3neo to generate pSox1KO4neo.



Screening strategy for correctly targeted ES cells by Southern blotting.

3.9 Targeting Sox1KO4neo in ES cells

The neo targeting vector pSox1KO4neo was introduced in E14TG2a ES cells by electroporation and clones were selected for hygromycin resistance as with the first vector (*Sox1* is not expressed in ES cells). Screening was carried out using the same probes and restriction digests as with the first vector, only in this case the sizes of the bands corresponding to the neo targeted clones were 9Kb for the 5' probe and 5Kb for the 3' probe (Figure 3.7). 180 colonies were picked, and of the 97 that were screened with both probes, two correctly targeted clones were identified (clones SIN4 and SIN5)(data not shown). The targeting frequency therefore is 2.06% in this experiment.

3.10 Chimera production and germline transmission

Targeted ES cells from clones 14, 46 and 53 (containing the *HyTK* cassette) and 46C from which the cassette has been excised, were injected into 3.5-day old C57BL/6 blastocysts by J. Nichols. Injected blastocysts were transferred to pseudopregnant foster mothers. Chimeras were identified by coat colour chimerism. Male chimeras were bred with MF1 females and grey coloured offspring (mixture of the 129/Ola chinchilla with the MF1 albino colours) were tested for presence of the *Sox1* targeted locus by Southern analysis with the 3' flanking probe and PCR (Figure 3.9). Germline transmission was only obtained from a chimera that did not carry the *HyTK* cassette (see Table 3.2). The analysis showed that 50% of grey pups inherited the targeted allele. Consistent with previous findings (Nishiguchi et al., 1998), heterozygous mice were phenotypically normal, and both males and females were fertile and transmitted the transgene at the expected frequencies. The line ($Sox1^{egfpAGS}$) was maintained by backcrossing heterozygous males to MF1 females. Homozygous animals were also generated by intercrossing two heterozygotes. These animals looked initially smaller than their wild-type littermates. The *Sox1* null animals had smaller eyes with opaque lenses indicating cataract, and started having seizures after about two months of age. These findings correlate well with the previously reported phenotype of *Sox1* -/- animals (Nishiguchi et al., 1998) (see Figure 3.8).

Figure 3.8 *Sox1*^{egfp} mice

Sox1 null animals appear smaller because of a hunched posture and have seizures. A *Sox1* null animal (arrow) is shown for comparison next to a heterozygous littermate. The *Sox1* null animal has had a seizure minutes before this picture was taken and was still trembling.

Table 3.2

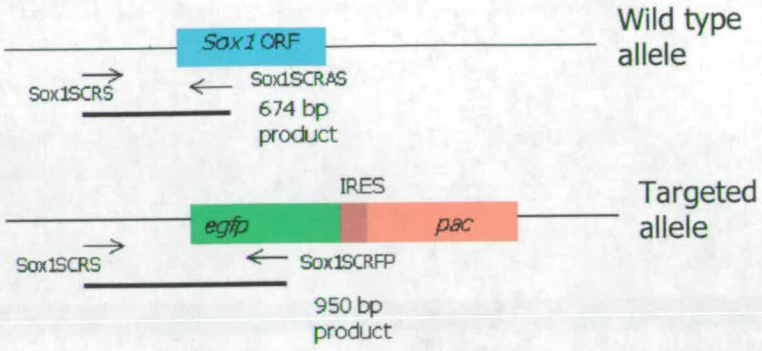
Summary of chimera production and germline transmission

ES cell clone	Blastocysts injected	Pups born	Chimeras (male)	Germline chimeras(pups born)
14	28	10	7 (4)	0 (150)
46	19	6	5 (4)	0(44)
53	83	8	6 (6)	0(0)
46C	16	3	3 (3)	1(9)

3.11 Summary

Sox1 has been targeted with two targeting vectors, containing different reporter/selection cassettes. In the case of the *egfp*-IRES-*pac* targeted clones, the selectable marker CMV*HyTK* used for selection of integration in ES cells has successfully been removed from one of the clones (clone 46). The targeting efficiency was similar for the two vectors (2-3%) which was anticipated as both vectors had identical sequence homology to the *Sox1* locus. The previous targeting of the *Sox1* locus reported (Nishiguchi et al., 1998), does not report clearly on the targeting frequency, so the results cannot be directly compared, but the homology they used was smaller than that shown here. Chimeras were generated from four clones targeted with the eGFP vector, one of which gave germline transmission. Both targeting vectors completely remove one copy of *Sox1*, replacing it with selectable markers. Heterozygous animals were generated. These animals are born in Mendelian ratios, are viable, apparently healthy and fertile. Animals lacking both copies of *Sox1* phenotypically resembled the previously reported *Sox1* –null mice (Nishiguchi et al., 1998) with microphthalmia, cataract and spontaneous seizures.

A



B

Sox1 SCRS: 5′ GACTTCGAGCCGAGAAGAGG 3′

Sox1 SCRAS: 5′ GCTTGCTGATCTCCGAGTTG 3′

Sox1SCRFP: 5′ GGCGGATCTTGAAGTTCACC 3′

C

Figure 3.9 PCR genotyping

A Diagram of the PCR strategy for screening tail genomic DNA. B Sequences of PCR primers used for genotyping. C A gel showing the result of one such PCR. Homozygous wild type and heterozygous animals shown as +/+ and +/- respectively (H_2O: water control).

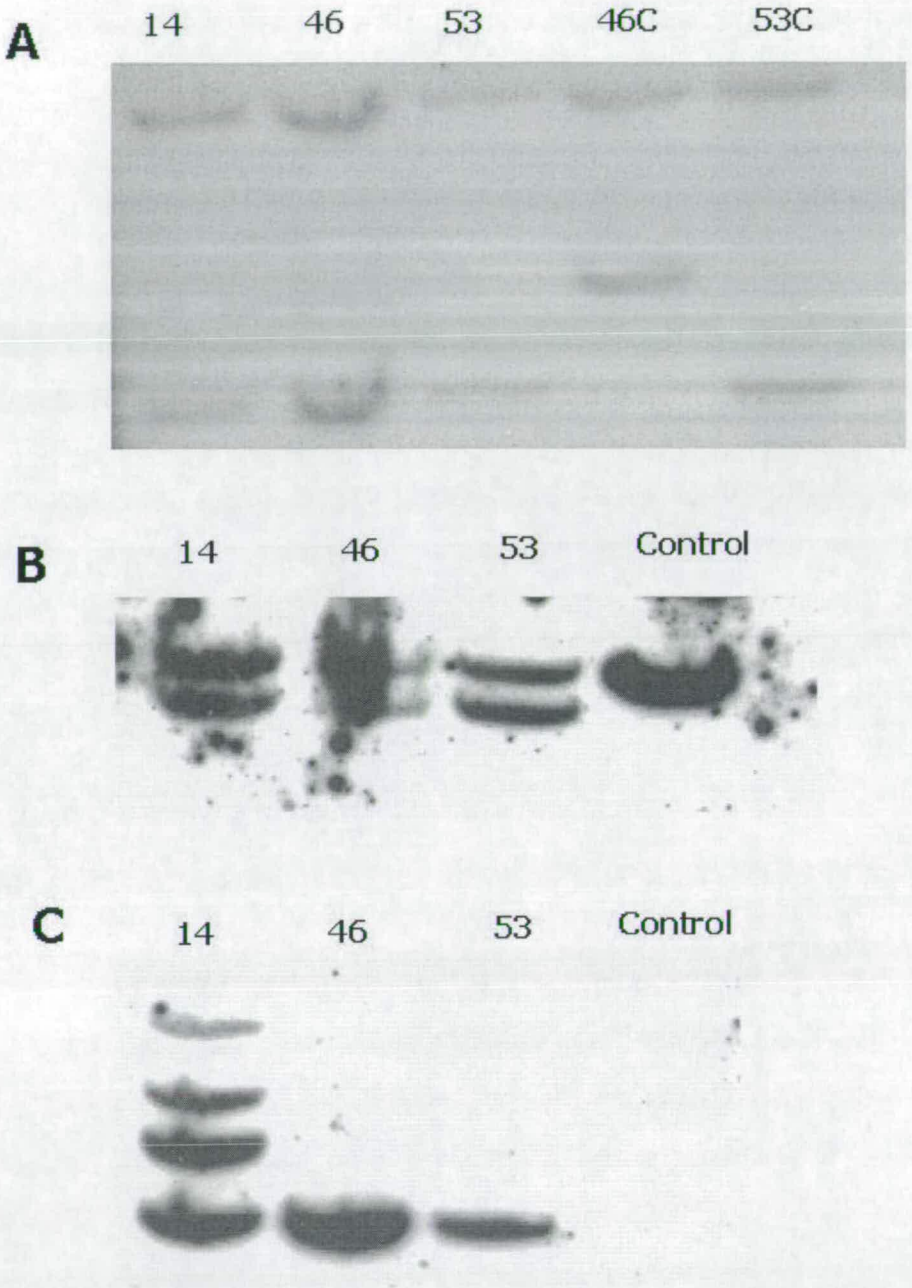


Figure 3.10 Southern blotting of targeted clones

Genomic DNA was prepared from ES cells, digested with appropriate restriction endonulease, ran on agarose gel, blotted and hybridised with the appropriate probe as described in materials and methods and above. A: 3' probe, B: 5' probe, C: internal EGFP probe

4 *Sox1* expression during development

4.1 Introduction

Targeting the reporter gene *egfp* into the *Sox1* locus as described in chapter 3, put *egfp* under the transcriptional control of the endogenous *Sox1* regulatory sequences. Therefore, sites of *Sox1* transcription can be visualised in transgenic animals by fluorescence microscopy for EGFP. *Sox1* expression during mouse development has been documented before, using an antibody (Pevny et al., 1998) and RNA *in situ* hybridisation (Wood and Episkopou, 1999). However, both of these studies only looked at expression during early development. Here, an investigation of sites of *Sox1* expression during mouse development is carried out, using EGFP to visualise it.

Expression described here is as visualised on animals resulting from backcrossing heterozygous founder males (mixed 129/Ola and MF1 genetic background) to MF1 females. In animals carrying *egfp*, the expression of the gene is entirely dependent on the activity of endogenous *Sox1* regulatory elements as described in chapter 3. Embryos were dissected at embryonic days (E) 7, 8, 9, 10, 11, 12, 14, 16 and 18 and observed whole-mount for EGFP fluorescence. Embryos older than E12 as well as adult animals were also cryosectioned and sections examined for fluorescence. Adult animals were transcardially perfused with 4% paraformaldehyde before embedding to reduce autofluorescence.

4.2 Expression before E9

Since *Sox1* expression has never been documented before E7, the earliest timepoint examined for Sox-EGFP expression was E7. However, no EGFP fluorescence was seen at that time point, despite prolonged exposures under UV with a digital camera. Fluorescence first becomes visible at around 8.25-8.5 days *post coitum* (dpc, Figure 4.1), at the early somites stage. Previous studies (Pevny et al., 1998; Wood and Episkopou, 1999) put the onset of *Sox1* expression slightly earlier (E7.5 and E8 respectively) when examined using an antibody or by RNA *in situ* hybridisation. This discrepancy is probably due to the time it takes for EGFP to fold properly to fluoresce, and/or the time needed for enough molecules of it to

accumulate in the cytoplasm to give appreciable fluorescence (Patterson et al., 1997).

Fluorescence initially appears at the middle part of the neural plate, contrary to the previous reports describing expression throughout the neural plate. This may be because the tissue is thicker there. By late day 8 $Sox1^{egfp}$ can be seen on all of the neural plate, being slightly more prominent at the middle of it where it begins to fold (Figure 4.1). This correlates well with the previous studies on *Sox1* expression (Pevny et al., 1998; Wood and Episkopou, 1999), which report uniform expression of Sox1 throughout the neural plate.

Sox1 has been previously associated with proliferating cells of the neural tube, being downregulated as cells exit mitosis and terminally differentiate (Pevny et al., 1998). By E9, neural induction has finished and the neural plate begins to fold and form the neural tube (Ybot-Gonzalez *et al.*, 2002). The neural folds/tube at this stage is composed mainly of proliferating precursors.

Under the influence of Sonic hedgehog (Shh) signals derived from the underlying notochord, cells at the ventral part of the neural tube (the region closest to the notochord) begin to differentiate into floorplate and upregulate *Shh*. Shh signals induce and repress different classes of homeodomain transcription factors at different concentrations. These transcription factors can function in a cell-autonomous manner to repress each other, thus establishing distinct domains of expression along the D-V axis of the neural tube (Briscoe *et al.*, 2000). These domains will later give rise to distinct classes of neurons, implicating Shh signalling in the patterning of the neural tube. Sox1-GFP appears weaker at the ventral midline of the neural tube at the later timepoints, suggesting exclusion of expression from the floorplate.

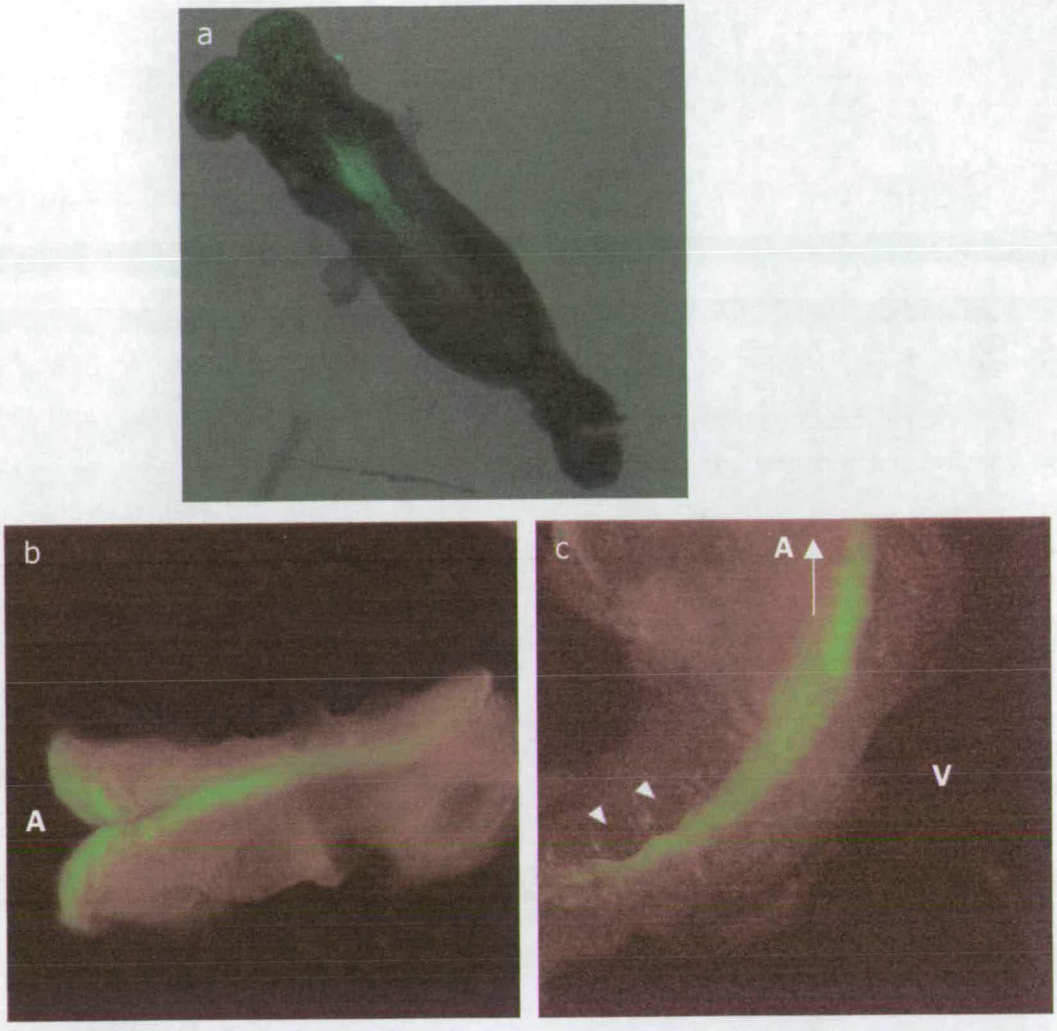


Figure 4.1 *Sox1*^{egfp} expression at E8.5

a: day 8.5 heterozygous embryo showing expression of the *egfp* transgene at the middle of the neural plate (anterior is top left). Sectioning would have been more informative on whether this localisation is due to tissue thickness or reflects genuine differences in expression b,c: Dorsal and lateral view respectively, of embryos at late E8.5. Fluorescence is seen throughout the neural plate/tube. Note that expression is excluded from the somites (arrowheads in c) (A: anterior, V: ventral).

4.3 Expression between E9-E13

After neural tube closure, non-neural ectoderm at the dorsal side of the neural tube expresses *Bmp* genes. BMPs produced by the ectoderm diffuse through the intervening mesenchyme and influence the dorsal part of the neural tube, causing the dorsal-most cells to form the roofplate (Lee *et al.*, 2000a). The roofplate cells start producing BMP signals to pattern the dorsal third of the neural tube (Timmer *et al.*, 2002). These opposing gradients of Shh and BMPs pattern the entire neural tube along its dorso-ventral axis.

Although no experiment was done to specifically address this point, Sox1-EGFP levels seemed to become weaker at the floorplate and roofplate of the neural tube (Figure 4.2 **c**,**d**). Cells in these regions are among the first to stop dividing in the developing nervous system concomitantly with the acquisition of their roles as signalling centres for neural tube patterning.

After mid-gestation, *Sox1* expression remains strong throughout the neuraxis, with the exception of the roofplate and floorplate (Figure 4.2). During these stages, patterning of the neural tube is established and distinct classes of neurons start to differentiate and migrate laterally. The proliferative cells become restricted in the layers closest to the ventricular area of the tube, and as cells terminally differentiate and migrate out, the neural tube thickens. In the $Sox1^{egfp}$ mice, GFP expression progressively becomes restricted to the ventricular layer. In the developing brain, expression is strongest at the telencephalic region after E10.5 (Figure 4.2 **a**,**b**).

In the developing eye, Sox1 is needed for γ-crystallin gene activation (Kamachi et al., 1995; Kamachi et al., 1998; Nishiguchi et al., 1998). EGFP expression first becomes evident at E11 in the lens. Previous reports put the onset of *Sox1* expression in the lens at E10.5, but the discrepancies can be explained by the different methods used for the detection (RNA *in situ* hybridisation *vs.* EGFP protein fluorescence). Expression of EGFP in the lens is maintained during this period (E9-E13).

In the brain around E12.5, expression is seen in regions of ongoing cell proliferation, most notably the cell layers abutting the ventricles and the forebrain vesicles which are rapidly expanding to make the cerebral cortex.

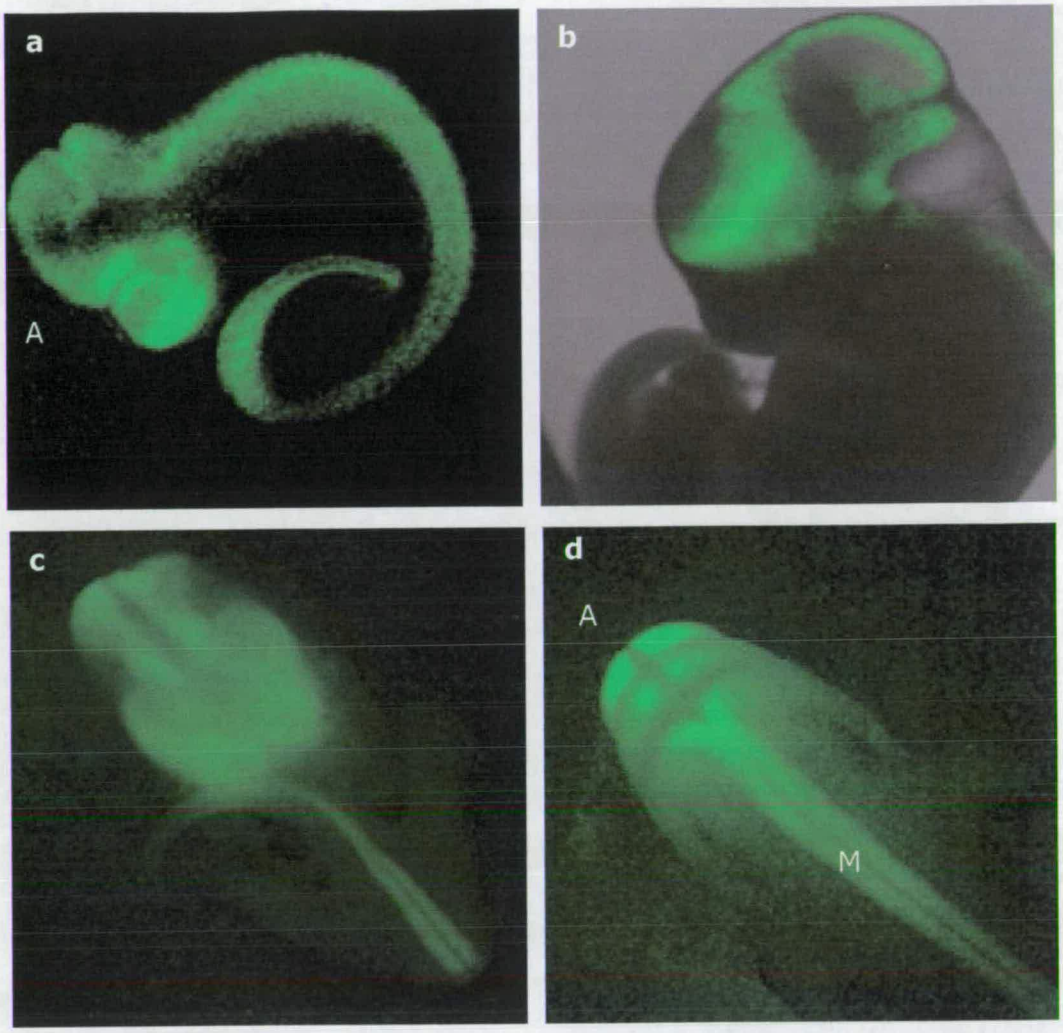


Figure 4.2 Sox1 expression between E11.5-E12.5

a: E10.5 embryo; expression seen throughout the neuraxis. Apparent punctate EGFP fluorescence is a photographic artefact b: E11.5 embryo, showing strongest expression in the brain. c,d: E12.5 embryos; *Sox1* expression is excluded from the roofplate and floorplate (M), but is seen along the entire length of the CNS (A: anterior, M: midline).

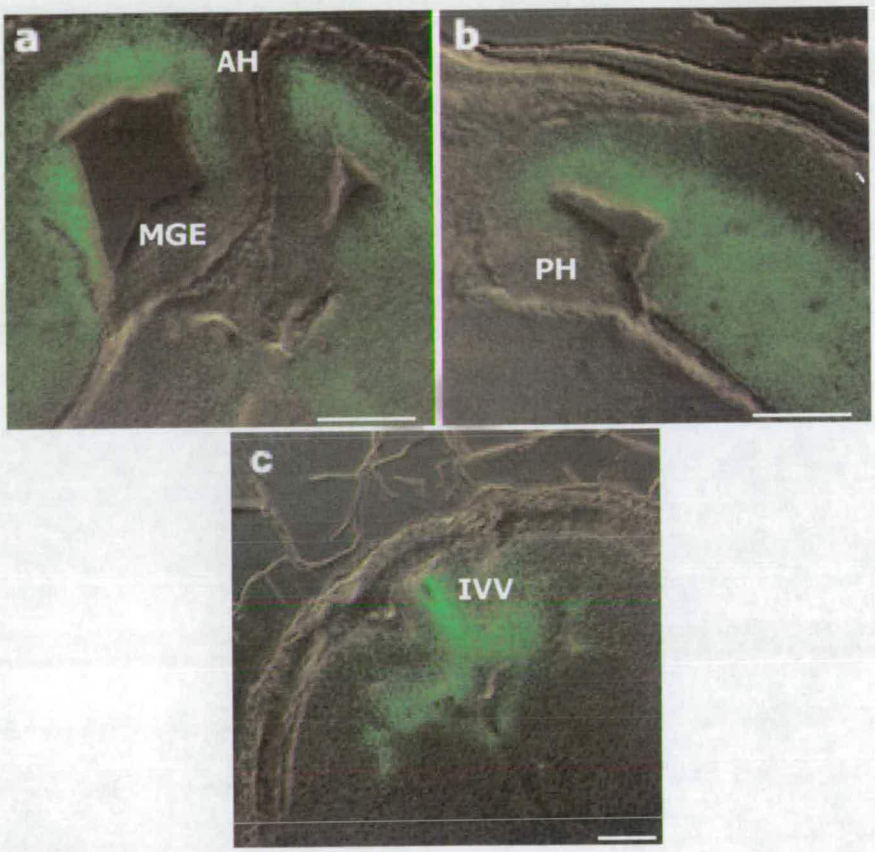


Figure 4.3 Sections of E12.5 *Sox1*^{egfp} heads

a: Horizontal section through the forebrain. AH: anterior horn of the lateral ventricle, MGE: Medial ganglionic eminence. **b**: Horizontal section at the level of the posterior horn (PH) of the lateral ventricle at E12.5. **c**: E12.5 horizontal section at the midbrain. Note expression around the fourth ventricle (IVV). Scale bars, 500μm.

4.4 Expression between E13-birth

After E12, expression becomes restricted to a thin ventricular zone of the spinal cord and the developing brain, being strongest in the forebrain and more specifically the lateral ventricles.

During this period, the brain expands by the production of neurons and glia and the basic neuronal circuitry is established. Proliferation is restricted to the areas adjacent to the ventricles, especially the third and lateral ventricles where the telencephalic vesicle expands to produce the cortex. Sox1-GFP expression is seen in the forebrain, especially around the lateral ventricles. After sectioning and examination of the slides for fluorescence, Sox1-GFP positive cells were only detected next to a ventricle. This expression becomes weaker at later stages, and at E18.5 is only seen in the subventricular layer of the lateral ventricle.

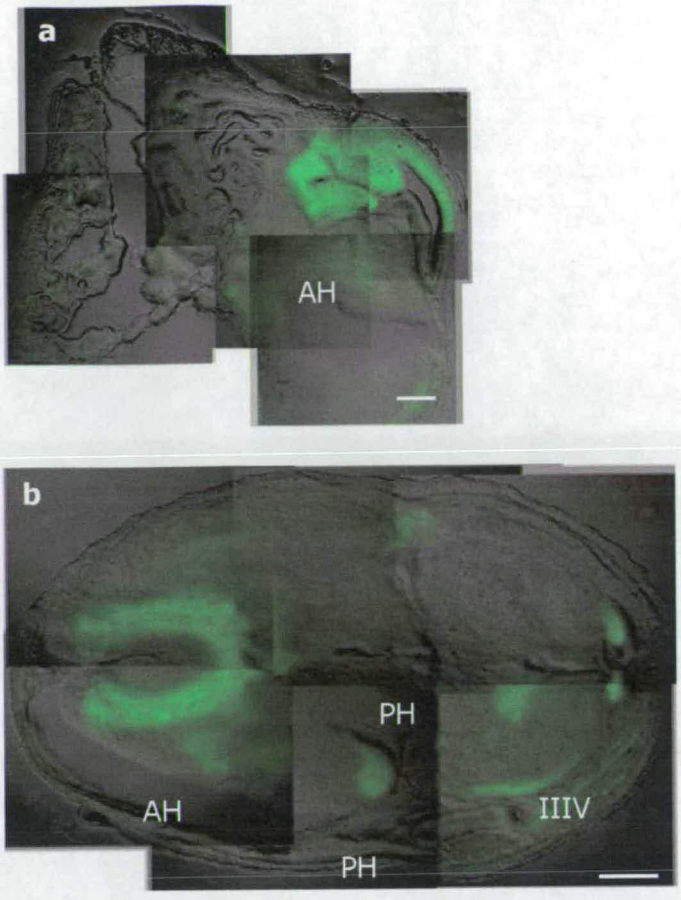


Figure 4.4 Sox1 expression at E14.5

a: Sagittal and b: horizontal sections through E14.5 head. Expression is strongest in cells adjacent to the lateral ventricle, in both anterior (AH) and posterior (PH) horn. Expression also seen adjacent to the third ventricle (IIIV). Scale bar, 500μm.

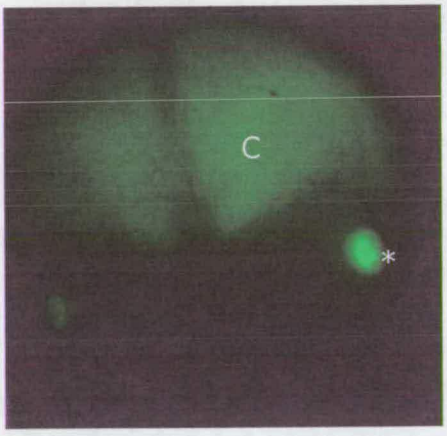


Figure 4.5 **Expression of Sox1-GFP at E15.5**

Frontal view of an E15.5 fetus head. Fluorescence is seen in the lens (*) and in the developing cortex (**C**).

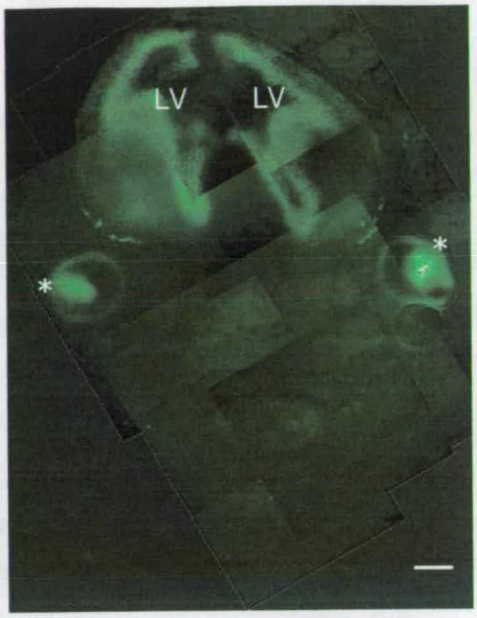


Figure 4.6 Coronal section at E12.5

Expression seen in the lens (*) and in cells around the lateral ventricles (**LV**). Scale bar, 500μm.

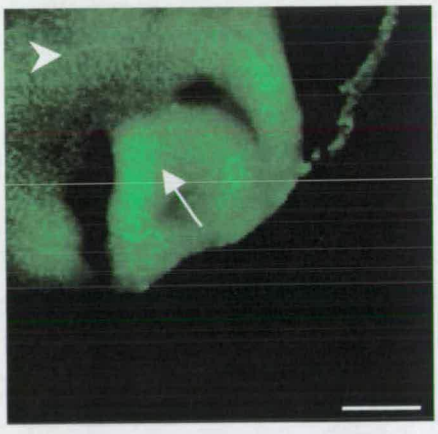


Figure 4.7 The $Sox1^{egfp}$ eye at E11.5

GFP image of the eye of a E11.5 embryo. Expression seen in the lens vesicle (arrow) and the presumptive retina (arrowhead). Scale bar, 500μm.

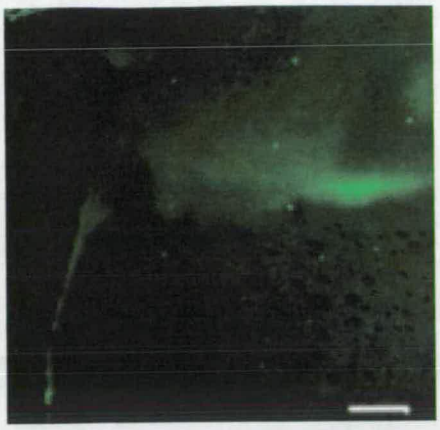


Figure 4.8 Sox1^{egfp} expression in an adult

Expression in the adult lateral ventricle subependyma. The section is at a level just above the ventricle, showing expression in the subependyma. Scale bar, 500μm.

4.5 Expression after birth

After birth, Sox1–GFP is seen at the lateral ventricle where onging neurogenesis is occurring (Altman, 1969; Doetsch *et al.*, 1999; Frisen *et al.*, 1998; Johansson *et al.*, 1999a; Johansson *et al.*, 1999b) (Figure 4.8). A limitation of the EGFP is that it is often difficult to distinguish between true fluorescence and autofluorescence. In the adult brain, strong autofluorescence is associated with myelin. Therefore, an apparent expression of *Sox1* in the cerebellum and the *corpus callosum* is attributed to autofluorescence since these are regions containing high numbers of myelinated axons.

EGFP expression is also seen along the rostral migratory stream, the route followed by migrating neuroblasts generated in the subventricular zone to the olfactory bulb (Doetsch et al., 1999).

4.6 Summary

Regions of Sox1 expression were visualised by fluorescence microscopy for the EGFP marker introduced into the *Sox1* locus. The sites of expression shown here correlate well with other studies of Sox1 expression carried out by *in situ* hybridisation (Kamachi et al., 1998; Wood and Episkopou, 1999) or

5 *Sox1* as a reporter of ES cell differentiation

5.1 Introduction

As mentioned before, the reason for developing the 46C cell line was to study ES cell differentiation to the neural lineage and purify cells of that lineage from other cell types coming through the differentiation protocol. The technique of lineage selection has been used previously in this laboratory for neural differentiation, by virtue of a *Sox2* reporter cell line (Li et al., 1998). However, the expression of *Sox2* is broader than that of *Sox1* (Pevny and Lovell-Badge, 1997; Wood and Episkopou, 1999) and it is also expressed in ES cells.

Therefore, a *Sox1* reporter cell line utilising EGFP was constructed as described in chapter 3. The main advantage of having EGFP as a reporter is that it enables observation in live cells and tracking the activity of the endogenous promoter over time in the same cell (timelapse imaging). Another advantage of the use of a vital label such as EGFP is that it permits the use of flow cytometry to accurately monitor levels of expression in a mixed population of cells, to quantify it and to sort cell populations based on the marker gene expression. I have used a combination of the above techniques to study ES cell differentiation and factors that affect it.

5.2 46C cells express *egfp* after differentiation

Embryonic stem cells can differentiate along the neural fate by a variety of methods (Bain, 1995; Fraichard et al., 1995; Kawasaki et al., 2000; Tropepe et al., 2001). It was described in the previous chapter that the expression of the EGFP reporter correctly followed the expression pattern described for *Sox1 in vivo*. *In vitro*, during ES cell differentiation, *Sox1* is expressed by neural progenitors (Li et al., 1998). This process begins after addition of retinoic acid during differentiation in embryoid bodies (Bain, 1995; Fraichard et al., 1995) on day 4 (J. Aubert, unpublished data).

In order to test the fidelity of the reporter gene expression during *in vitro* ES cell differentiation, 46C cells were subjected to embryoid body differentiation and analysed at different timepoints for green fluorescence. No green cells were detected in undifferentiated populations of 46C ES cells or in embryoid bodies

before treatment with retinoic acid, in agreement with RT-PCR data suggesting that *Sox1* is not expressed in these cells (J. Aubert, unpublished). After retinoic acid treatment on day 4 of EB differentiation, some of the cells started to activate the *Sox1* locus and consequently fluoresced green (Figure 5.1). By day 8, a significant portion of the cells in each embryoid body were EGFP positive.

5.3 Monolayer differentiation

ES cell differentiation *in vitro* to the neural lineage is initiated via poorly understood interactions in serum-containing medium (Fraichard et al., 1995; Okabe et al., 1996), multicellular aggregates (Tropepe et al., 2001) and/or co-cultures (Kawasaki et al., 2000) often in the presence of morphogens (e.g. RA) (see section 1.3). These protocols are hindered by varying degrees of heterogeneity in terms of their efficiency in generating neural cells often caused by the use of non-defined media containing as much as 10% serum (Strubing et al., 1995), the uncharacterised interactions between aggregated cells (Tropepe et al., 2001) or the use of stromal cells to induce the differentiation (Kawasaki et al., 2000).

These conditions preclude a systematic dissection of the factors required for neural lineage specification of ES cells because of their variability and qualitative nature. In most cases it is virtually impossible to assess the effect of an added factor on the differentiation efficiency until the experiment has finished and the cells fixed and processed immunocytochemically. In other cases, where relatively homogeneous populations of cells are obtained by differential viability in a chemically defined medium (Lee et al., 2000b), the initial differentiation steps are poorly characterised and the selection process amplifies a rare fraction of the starting cell population.

A novel protocol for the differentiation of ES cells to the neural lineage in fully defined, serum-free monolayer culture was recently developed in the laboratory (Ying *et al.*, submitted) (see section 1.3.4). The entire process can be monitored at the level of individual cells. 46C cells were used to analyse the commitment of cells during the differentiation process. The first GFP positive cells appeared in the cultures 3-4 days after plating and shortly after this the majority of cells expressed EGFP. The acquisition of GFP fluorescence happens asynchronously between colonies. This indicates that neural commitment is not the result of an enviromental

cue, rather a stochastic process. As the cells progressively differentiate into neurons and glia, they downregulate *Sox1* and consequently lose GFP fluorescence. The cells are often arranged into rosette-like colonies, in which the majority of cells are expressing EGFP (Figure 5.2).

The growth kinetics of cells undergoing such differentiation were measured by cell counts at 9 hours after plating and each day thereafter for 9 days. The results indicate that despite an initial reduction in cell numbers by 19% attributed to plating efficiency in serum-free conditions, the cell numbers increase exponentially during this period of culture (Figure 5.3). The plot of cell numbers over time in a logarithmic scale produces points with strong linear regression (r^2=0.931, P<0.001).

Since the differentiation process is happening in adherent monolayer, it is possible to observe the morphological changes of the cells as they differentiate. This is contrasting to EB and to PA-6 differentiation where differentiation happening in cell aggregates or three-dimensional colonies.

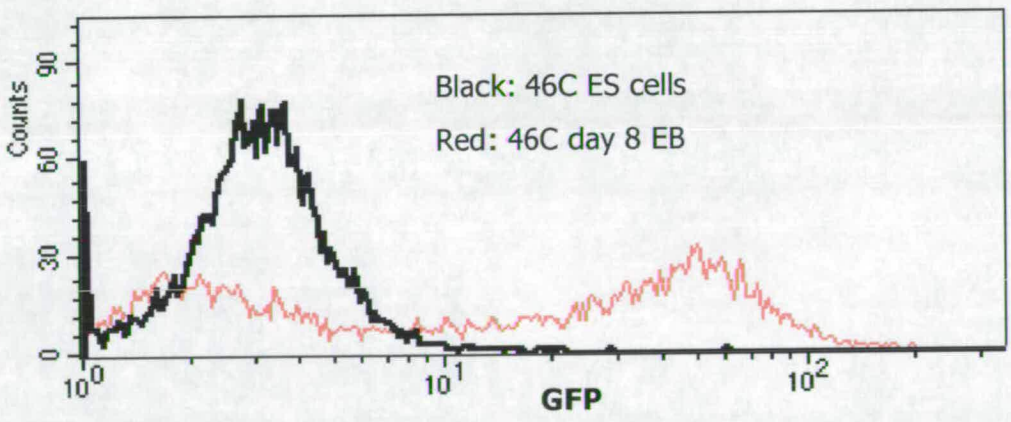


Figure 5.1 Fluorescence after retinoic acid treatment of 46C embryoid bodies

46C ES cells were differentiated as embryoid bodies for 4 days in medium without LIF, and for a further 4 days in medium without LIF supplemented with 10^{-6}M all-trans retinoic acid. On day 8, EBs were trypsinised and analysed by flow cytometry. GFP scale is representing fluorescence intensity (arbitrary units).

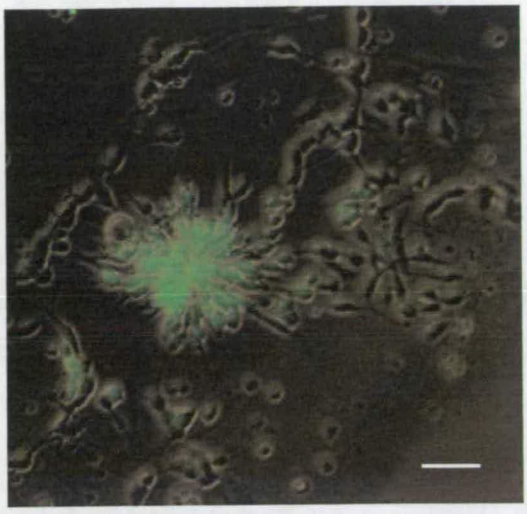


Figure 5.2 Rosette of 46C cells

A rosette of GFP positive cells on day 5 of monolayer differentiation. Scale bar 50μm.

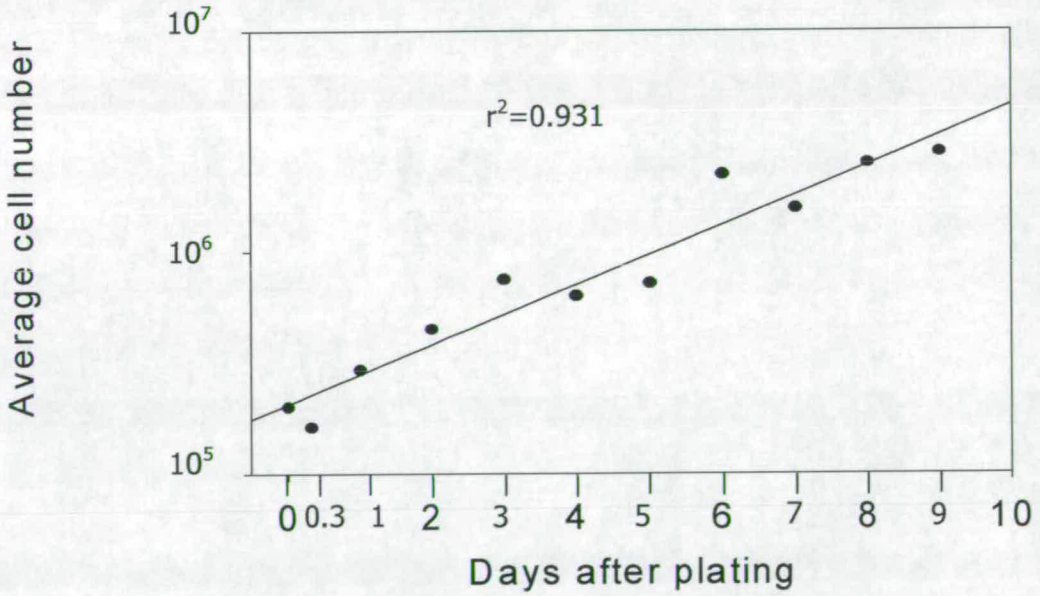
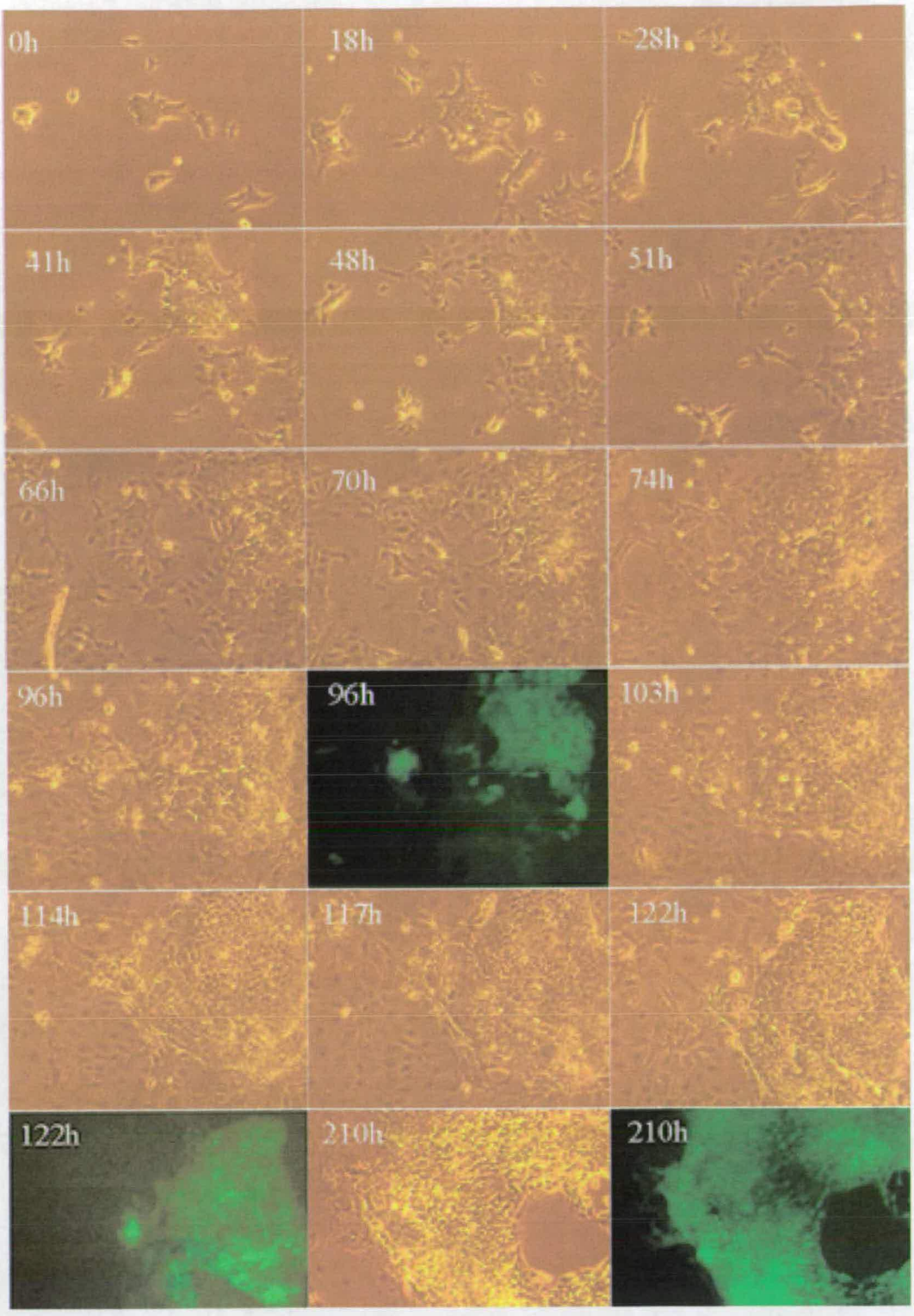


Figure 5.3 **Culture growth during monolayer differentiation**

Plot of cell numbers during monolayer differentiation. Points are averages of two experiments performed in triplicate. Regression significant at $P<0.001$.

Figure 5.4 Timelapse study of 46C monolayer differentiation

Consecutive photomicrographs of the same field were taken at regular intervals. Pictures taken in collaboration with Dr. QiLong Ying. ES cells were plated at clonal density in ES cell culture medium for 24 hours, to allow them to attach. The medium was then changed to N2B27 and a selected field was observed every 2-4 hours and a picture taken. Under these conditions, alternative differentiation is more pronounced, presumably due to the presence of traces of serum during the early stages of the differentiation process. The flat, large cells apparent in this timelapse experiment cannot be responsible for the differentiation, as they do not appear in most of the differentiation experiments described here. The observation of the cells every 2-4 hours was necessary to avoid killing the culture, but presents the limitation that individual cell divisions cannot be observed. Scale bar 50μm.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The characteristic ES cell morphology is progressively lost, as cells initially become flatter and resemble an epithelium, later to be replaced by smaller, bipolar cells that are often arranged in a rosette structure and later by neurons and glia that downregulate the EGFP reporter (Figure 1.4 for timelapse images of the same colony).

In order to quantify the conversion of ES cells to EGFP positive presumptive neural precursors, 46C cells were differentiated in monolayer culture and each day analysed by flow cytometry for EGFP expression. Undifferentiated ES cells do not show any fluorescence before the differentiation protocol starts, so the marker region for GFP+ was set to exclude 99% of the initial ES cell population.

In the first two days after plating, less than 10% of cells became positive for GFP. On day 3, the proportion of live cells that were within the marker region had increased to around 42%, and a day later to 75%. During days 5, 6 and 7 the proportion of GFP positive cells remains approximately stable around 75-80%, before starting to decrease as cells turn off *Sox1* and terminally differentiate to GFP negative neurons and glia (Figure 5.5). By day 10, only around 47% of live cells remain EGFP positive. These flow cytometry data are consistent with observations made by fluorescence microscopy during the same period.

Given that the acquisition of EGFP expression is asynchronous between colonies and that differentiation to neurons and glia is accompanied by downregulation of *Sox1* in the embryo (Pevny et al., 1998), the number of EGFP positive cells at any time point is likely to be an underestimate of the total incidence of neural determination. However, not all of the cells in a culture become EGFP positive and some remain undifferentiated ES cells, implying that alternative differentiation programmes and some self-renewal are at work in these monolayers. The different cell types in the culture appear to be proliferating with similar rates, although proliferation rates were not measured for any experiment.

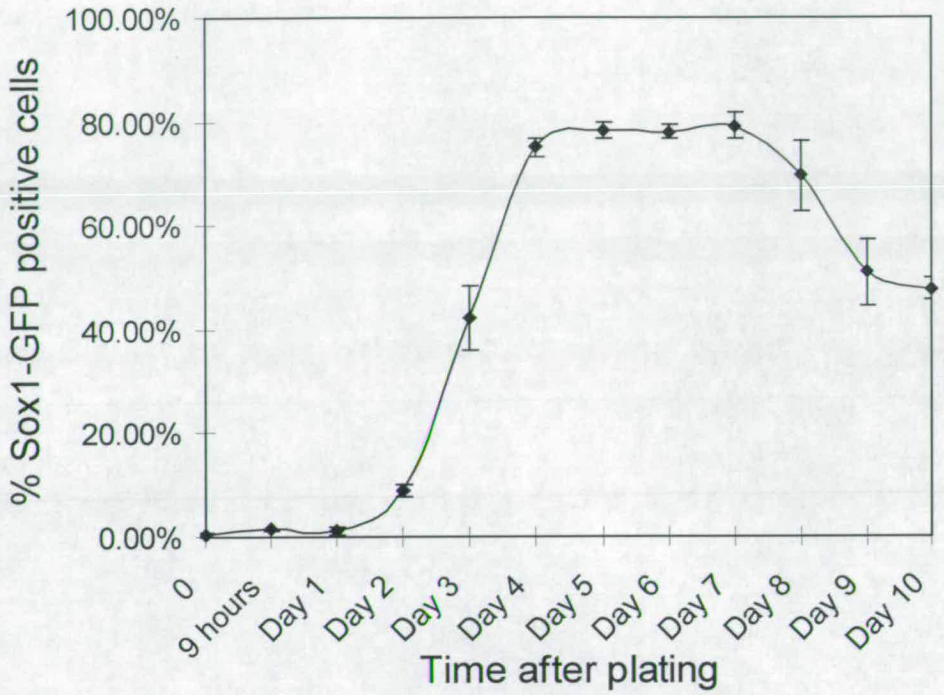

Figure 5.5 Quantitation of monolayer differentiation of 46C cells

Points are averages of two experiments performed in triplicate.

5.4 Effect of exogenous factors on the monolayer differentiation

The impact of candidate regulatory factors on neural determination in monolayer differentiation was assessed. Factors to be tested were added at the time of plating and replenished every two days when media were changed. The proportion of ES cells (for example, by SSEA1 antibody staining) was not measured for these experiments.

5.4.1 LIF

Leukemia inhibitory factor is a cytokine that acts through the gp130- LIF receptor heterodimer (Gearing and Bruce, 1992; Stahl *et al.*, 1993). It is routinely used on ES cell cultures to maintain the cells in an undifferentiated state in the absence of any feeder cells (Smith et al., 1988; Williams et al., 1988). This is

achieved by signalling downstream of the LIF receptor/gp130 heterodimer receptor and activation of the transcription factor STAT3 (Matsuda et al., 1999; Niwa et al., 1998). Recently, there has also been a report that LIF is essential for the survival/proliferation of a primitive neural precursor derived directly from ES cells *in vitro* or the pluripotent epiblast *in vivo* (Tropepe et al., 2001).

Addition of LIF (100 units/ml) during monolayer differentiation suppressed the emergence of GFP positive cells (Figure 5.6) and resulted in increased number of ES-like colonies. This is in agreement with the normal neuroectoderm formation in LIF-receptor knockout embryos (Li et al., 1995), with the established action of LIF in promoting self-renewal of undifferentiated ES cells (Smith et al., 1988; Williams et al., 1988), and with the inhibitory effect of LIF on stromal cell-induced differentiation (Kawasaki et al., 2000).

During normal ES cell culture, LIF is a strong supressor of mesodermal and endodermal differentiation (Smith, 1991). However, an appreciable proportion of GFP positive cells were obtained from LIF-treated monolayers (around 50% compared to controls, around 35% of all cells), which may indicate that LIF receptor signalling does not block the neural lineage very efficiently and/or that some factor present in serum normally collaborates with LIF.

The antagonist for the LIF receptor hLIF-05 (Vernallis, 1997) (at a concentration determined to completely block the effect of 10 units/ml of LIF) had little effect on the monolayer differentiation, suggesting that commitment during monolayer differentiation is not dependent on LIF signalling.

5.4.2 BMP-4

In fish and amphibian embryos, the naïve ectoderm becomes epidermal or neural depending on the presence of signals from the underlying mesoderm. Ventral mesoderm secretes BMP-4, a TGFβ-like growth factor, which causes the unspecified ectoderm to assume an epidermal fate. Dorsal mesoderm secretes antagonists of BMP, neutralising the activity of BMP-4 produced by the ectoderm and by the ventral mesoderm. Naïve ectoderm that receives no BMP signal assumes a neural fate. This is the proposed "default" model for neural induction which proposes that it is the absence of inductive signals that determines the neural fate of ectoderm

(Wilson and Hemmati-Brivanlou, 1995; Wilson and Hemmati-Brivanlou, 1997)(see section 1.2).

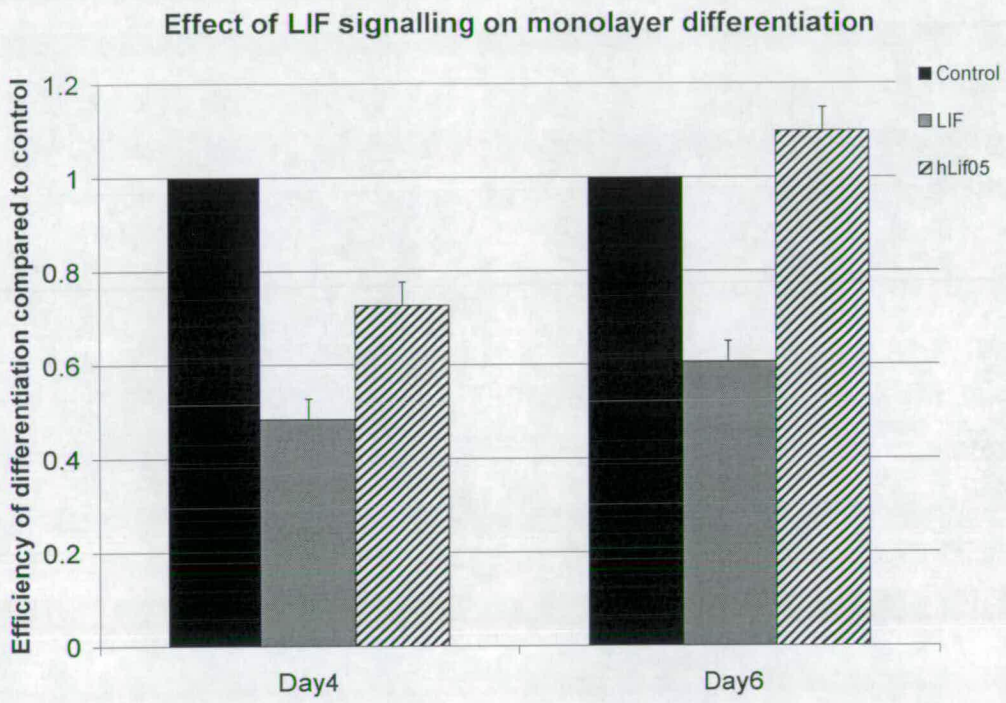

Figure 5.6 Effect of LIF signalling on monolayer differentiation

ES cells were plated at $2x10^5$ cells per gelatinised 6cm plate in N2B27 in the presence of 100U/ml human recombinant LIF or the LIF receptor antagonist hLif05 at a concentration to inhibit 10U/ml LIF. Medium was changed every two days with fresh growth factors and inhibitors. At days 4 and 6 cells were dissociated and analysed by flow cytometry. The proportion of EGFP positive cells in each condition is plotted normalised against control cultures. Each bar represents two independent experiments performed in triplicate, and the controls averaged 72%. Bars: Standard deviation.

In vitro studies on several ES cell differentiation systems have established a negative role for BMP-4 in neural induction of these cells (Kawasaki et al., 2000; Rohwedel *et al.*, 1998; Tropepe et al., 2001; Wiles and Johansson, 1999). When added to the monolayer differentiation, 50ng/ml BMP-4 completely suppressed the emergence of Sox1-GFP positive cells (Figure 5.8). This happened while there was an increase in the cell numbers and alternate cell morphologies appeared (Figure 5.7). However, no cells with overt neuronal morphology were generated, consistent with the notion that BMPs suppress neural differentiation of naïve ectodermal cells. This BMP-4 effect was also seen at lower concentrations (10ng/ml and 5ng/ml) (not shown). The cell morphologies of BMP-4 treated monocultures resembled those of mesendodermal cells appearing in ES cell cultures upon withdrawal of LIF (Figure 5.7). RT-PCR analysis has shown that mesodermal markers are upregulated in such cultures, indicating that BMP directs a non-neural differentiation pathway of ES cells under such conditions (M Li, unpublished).

5.4.3 Noggin and Chordin

These are BMP antagonists secreted by the organiser during gastrulation. In *Xenopus* embryos, treatment of animal caps with these factors causes differentiation to neural tissue. In a whole *Xenopus* embryo, Chordin and Noggin have dorsalising activities. Single targeted mutations in either of these genes in the mouse results in normal gastrulation and neural induction, with effects seen later during embryogenesis. The compound mutants however have a range of phenotypes, commonly lacking anterior neural structures, suggesting a redundancy in the action of the two molecules (Bachiller et al., 2000) in anterior nervous system induction.

In an attempt to increase the proportion of GFP positive cells, Noggin or Chordin were added to the cultures. At the concentrations tested (0.1μg/ml, Noggin also at 0.5μg/ml), these factors consistently had no effect on the differentiation efficiency (Figure 5.8).

To test if the concentrations of Noggin and Chordin used were effective, cells were plated in the presence of 10ng/ml BMP-4 and 0.1μg/ml Noggin or Chordin in N2B27 medium. On days 4 and 6, cells were analysed by flow cytometry for Sox-GFP fluorescence. Noggin completely relieved the negative effect of BMP-4, whereas

Chordin did not (Figure 5.8). This effect can possibly be explained by the different affinities Noggin and Chordin have for BMP-4 (Piccolo et al., 1996; Zimmerman et al., 1996) or by increased stability of the recombinant Noggin/Fc chimera compared to Chordin. Moreover, the concentration of exogenous BMP-4 used (10ng/ml and 50ng/ml) is probably much higher than the levels likely to be present in the cultures.

Addition of Noggin and Chordin alone or in combination to ES cells plated at similar conditions to those used for monolayer differentiation but in the presence of serum was not sufficient to convert them to a *Sox1* positive phenotype (not shown). This suggests that serum contains other factors besides BMPs that inhibit neural differentiation.

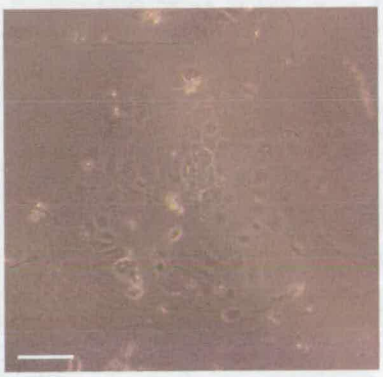

Figure 5.7 Differentiated cell morphology after BMP treatment

Morphology of 46C culture treated with BMP-4 for 6 days during monolayer differentiation. Scale: 100μm.

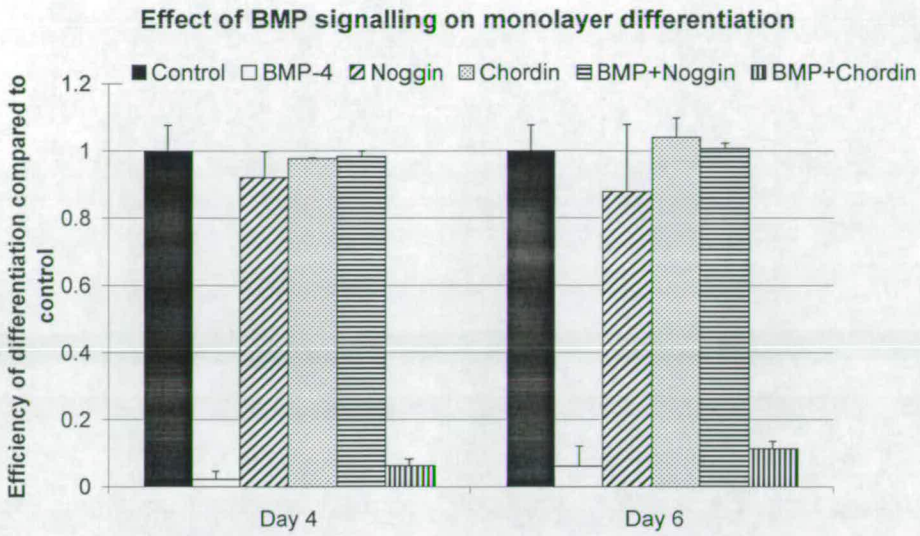

Figure 5.8 Effect of BMP signalling on monolayer differentiation

ES cells were plated at $2x10^5$ cells per gelatinised 6cm plate in N2B27 in the presence of 50ng/ml BMP-4 and/or 0.1μg/ml Noggin or Chordin. Medium was changed every two days with fresh growth factors. At days 4 and 6 cells were dissociated and analysed by flow cytometry. The proportion of EGFP positive cells in each condition is plotted normalised against control cultures. Each bar represents two independent experiments performed in triplicate, except for Noggin and BMP (5 experiments in triplicate), and the controls averaged 57%. Bars: Standard deviation.

5.4.4 FGF-2

Recent experiments in the chick have suggested that neural induction in that organism is more complex than the default mechanism described for amphibians and fish (Streit et al., 2000). This theory has postulated that neural differentiation happens in four steps: Competence, specification, commitment and differentiation (Wilson and Hemmati-Brivanlou, 1997). Neural competence is acquired before the onset of gastrulation, when no BMPs or BMP antagonists are being expressed. Competent cells have the ability to become neural precursors if exposed to the right combination of signals. Specification occurs when cells receive the signals to become neural, but will still respond to signals that repress a neural character. When cells become committed, they will progress to become neuronal or glial even in the presence of signals that repress a neural character. Differentiation is the final stage in which cells exit the cell cycle and terminally differentiate. One factor that

has been proposed to act at the competence stage and is therefore required for neural induction is signals from the FGF family.

In an experiment trying to address whether the efficiency of neural differentiation can be enhanced by such FGF signals, 20ng/ml FGF-2 (with or without 5μg/ml heparin) was added to the cultures. The result was a slight suppression of neural differentiation as measured by GFP fluorescence (Figure 5.9) accompanied by an apparent increased growth of the culture (not shown).

5.4.5 SU5402

Experiments in the chick done to investigate the role of FGF signalling in neural induction revealed that blocking FGF receptor signalling causes medial epiblast cells (which would normally give rise to neural tissue) to become epidermal. This is in agreement with a proposed role of FGFs in promoting competence of the ectoderm to respond to neuralising signals. These experiments were done using a pharmacological inhibitor of the FGF receptor family. This compound, SU5402, occupies an ATP binding site on the kinase domain of the receptor molecule and blocks activation of downstream pathways (Mohammadi *et al.*, 1997).

Addition of SU5402 to monolayer differentiation at 5μM causes a sharp reduction in the proportion of GFP positive cells (Figure 5.9) with no overt effect on the viability or proliferation (although the proliferation rate was not measured). This result is already apparent at day 3 of monolayer differentiation, suggesting that the action of SU5402 is at the level of neural specification rather than survival or expansion of neural precursors. Lower concentration of the compound (2μM), reported to inhibit neural induction in chick epiblast explants (Wilson et al., 2001) has no effect on the differentiation efficiency (Figure 5.9), whereas a high dose (20μM) killed the cultures (not shown). Furthermore, co-addition of Noggin (Figure 5.9) or Chordin (not shown) with 5μM SU5402 did not rescue the effect of SU5402. The specificity of the effect of SU5402 on FGF signalling was verified from experiments using a dominant negative FGF receptor in this system which gave similar results to those obtained with SU5402 (Ying *et al.*, submitted).

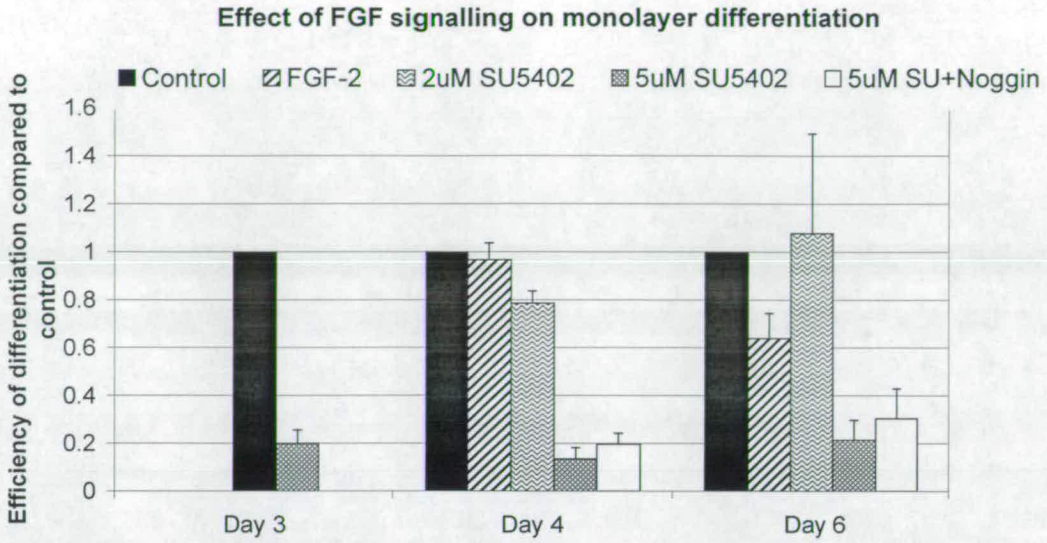

Figure 5.9 Effect of FGF signalling on monolayer differentiation

ES cells were plated at $2x10^5$ cells per gelatinised 6cm plate in N2B27 in the presence of 20ng/ml FGF-2, 2μM or 5μM SU5402. Medium was changed every two days with fresh growth factors. At days 4 and 6 cells were dissociated and analysed by flow cytometry. The proportion of EGFP positive cells in each condition is plotted normalised against control cultures. Each bar represents two independent experiments performed in triplicate, except for 2μM SU5402 (4 experiments in triplicate) and 5μM SU5402 (5 experiments in triplicate), and the controls averaged 61%. Bars: Standard deviation.

5.5 *Sox1* positive cells are free from ES cells

To verify that *Sox1*-GFP cells are free from ES cells, 46C ES cells were allowed to differentiate in monolayer for 4 days, then were dissociated by mild trypsin treatment and resuspended in PBS containing 10% FCS. The cells were then passed through a 35μm mesh filter to remove cell clumps and analysed by flow cytometry for green fluorescence (Figure 5.10**a**). Appropriate gates were set to cleanly separate GFP positive (58.5%) from GFP negative (17.5%) cells, and then cells were sorted into two tubes according to their GFP fluorescence. RNA was extracted from these populations and after reverse transcription, PCR was performed using specific primers for *Sox1, egfp, Oct-4* and *β-actin*.

As anticipated, *egfp* mRNA was expressed strongly in the GFP positive population, along with *Sox1*, whereas they were only expressed at a very low level by the GFP negative population (Figure 5.10**b**). The expression of *Sox1* by the GFP negative population is probably reflecting the onset of expression of *egfp* when not enough protein is present in the cytoplasm to confer green fluorescence to the cell. Importantly, the GFP positive fraction did not express the essential ES cell transcription factor *Oct-4*, in contrast to the negative population, in which the *Oct-4* mRNA was abundant.

This indicates that the *Sox1* positive cells are free from contaminating ES cells. Sorting positive from negative populations at this timepoint (day 4) results in poor viability of the *Sox1* positive fraction. However, when sorted into ES cell medium supplemented with LIF, GFP negative cells gave a high number of ES cell-like colonies that stained positive with an antibody against Oct-4. Few ES cell colonies were observed in the sorted cultures of GFP positive cells, and contamination of the sort can probably account for this.

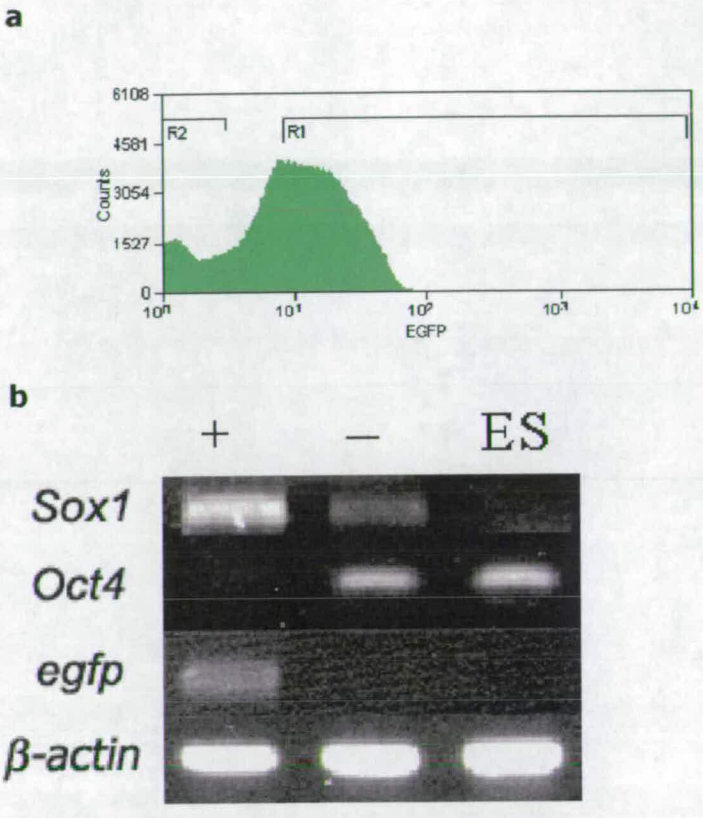

Figure 5.10 *Sox1* positive cells are free from ES cells

(a): FACS profile of the differentiating culture at day 4. Dead cells and cellular debris were excluded from the analysis based on their light scatter characteristics. Gates separating the positive (R1; 58.5%) from the negative (R2; 17.5%) population are shown.(b): RT-PCR analysis of sorted positive (+) and negative (-) cells from (a) and ES cells growing in standard ES cell medium (ES) Cells with intermediate levels of GFP fluorescence probably represent the tails of the distribution curves for the positive and negative populations, although some intermediate cell type with low *Sox1* expression cannot be excluded .

5.6 Discussion

Monolayer differentiation is a novel differentiation protocol for neural cell generation from ES cells. The main advantages over other ES cell differentiation systems are: completely defined media, no aggregation or co-culture of the cells, and high efficiency. Neural differentiation of ES cells in completely defined culture medium has been described in the past (Tropepe et al., 2001; Wiles and Johansson, 1999), but the frequency of neural determination is very low and the differentiation is happening in cell aggregates. An efficient method for generation of neural cells from ES cells in defined culture media without aggregation has also been described, but this depends on the co-culture of ES cells with stromal cells (Kawasaki et al., 2000). The nature of these interactions is still unclear, so hindering the study of lineage commitment requirements. Moreover, in light of recent observations suggesting that ES cells spontaneously fuse to other cells when co-cultured, such mixed cultures become less attractive to study cell lineage specification (Terada *et al.*, 2002; Ying *et al.*, 2002). A further advantage of monolayer differentiation over previously described protocols for ES cell differentiation is that nutrients and other media factors are equally available to all the cells in the culture, making the cultures much more homogeneous.

During the course of monolayer differentiation, ES cells efficiently convert to neural cells expressing *Sox1*. The conversion happens asynchronously through the cultures, suggesting a stochastic mechanism for neural determination. Expression of *Sox1*-egfp starts in small clusters of cells and is very rarely seen in isolated cells. This indicates that signalling between cells in a colony may be an important factor in the differentiation process. However, the efficiency of the differentiation is sensitive to variation in cell number; high plating density results in better plating efficiency, but the proportion of *Sox1*-egfp positive cells generated is lower. Low plating density compromises the initial survival of the cells, presumably due to lack of autocrine trophic signals and the absence of serum. The surviving cells are however more efficiently converted to *Sox1* positive neural precursors, a fact which indicates that some factor(s) produced by the ES cells (e.g. LIF) negatively regulate neural differentiation and promote survival and ES cell maintenance. The presence of background cell types in monolayer culture as seen in figure 5.4 is not believed to

be the cause of the differentiation due to production of some factor, as their number varies between experiments and their presence does not correlate with an increased differentiation efficiency. Indeed, in several experiments no background differentiation was observed, and the efficiency of differentiation was high (above 65%), indicating a negative effect of such cells on the efficiency of the differentiation.

The emergence of *Sox1*-egfp positive cells is progressive in differentiating cultures. At day 2, only a few colonies contain *Sox1* positive cells, but a day later both the number of positive cells in the colonies and the number of positive colonies increase, contributing to an overall increase in the proportion of *Sox1* positive cells. The next three days (days 4, 5 and 6) the proportion of *Sox1* positive cells reaches its maximum; this is accomplished by the acquisition of *Sox1* expression of yet more colonies and a proliferation of *Sox1* expressing cells while maintaining *Sox1* expression. Importantly, not all cells in culture express *Sox1* at any stage, indicating the acquisition of alternative non-neural fates under the same conditions as well as persistence of ES cells.

Since no exogenous mitogen is present in the culture medium, cells start to differentiate according to their intrinsic programme. After day 4 neurons start appearing in the cultures. Neurons are *Sox1* negative as they are non-proliferative and Sox1 is confined to mitotic cells (Pevny et al., 1998). As the numbers of neurons increase and Sox1 positive cells start differentiating and turning off *Sox1*, the proportion of egfp positive cells starts to decline after day 6. By day 10 after the start of the differentiation, the proportion of cells still expressing egfp is less than 50%. At later timepoints the number of neurons continues to rise and the proportion of *Sox1* positive precursors to decline.

The number of cells present in the cultures was counted to discount the possibility that this protocol depends on the amplification of rare cells present in the initial ES cell cultures. A previous publication reporting ES cell differentiation in serum-free medium reported a dramatic reduction in cell number after initial plating (Tropepe et al., 2001). That report was based on observations made on <0.2% of the initially plated cells; it is therefore conceivable that those cells were already committed to a neural fate, having spontaneously differentiated in the starting ES cell culture. In our system, the total number of cells increases exponentially,

indicating that ES cell lineage commitment in serum free conditions is a general property of ES cells rather than that of a rare subpopulation.

When the components of the medium were dissected to identify the neural inducing factor(s), it was discovered that several of the protein components of the N2B27 medium are not necessary for the differentiation. Transferrin is necessary for cell viability and insulin assists with cell attachment, but can be omitted. These results resembled the default model for neural induction first proposed for amphibians (Weinstein and Hemmati-Brivanlou, 1997). There have been some reports indicating that ES cells adopt neural fate in a default-like fashion (Tropepe et al., 2001; Wiles and Johansson, 1999), however the differentiation involved formation of cell aggregates, precluding systematic analysis of the lineage restriction process and the signals that govern it.

To address this hypothesis, proteins thought to be important for neural induction were added or inhibited during the course of monolayer differentiation. BMP-4 caused a strong suppression of the emergence of *Sox1* positive cells, consistent with its role as an inhibitor of neural fate during gastrulation stages *in vivo*. In frog and chick experiments, BMPs are thought to promote epidermal over neural fate of naïve ectodermal cells. Interestingly, preliminary evidence suggests a mesodermal rather than epidermal differentiation upon BMP treatment during this differentiation.

ES cells express *Bmp4* as well as BMP receptors (Roelen *et al.*, 1997), so I hypothesised that endogenous BMP signalling might be limiting the efficiency of differentiation. However, addition of Noggin or Chordin to the medium at the time of plating did not significantly alter the proportion of *Sox1*-egfp cells generated. This could be because ES cells express high levels of another inhibitor of BMP signalling, Follistatin (QiLong Ying, unpublished). This result indicates that autocrine BMP signalling is not a limiting factor for neural specification during monolayer differentiation. Addition of Noggin and Chordin either alone or in combination to 46C ES cells growing in serum containing medium without LIF did not result in the generation of *Sox1*-egfp positive cells. This result suggests that the apparent default differentiation of ES cells to neural cells is inhibited in the presence of serum by mesoderm-inducing factors other than BMPs.

Addition of LIF to the cells causes a reduction in the proportion of egfp positive cells of ~50% compared to the untreated control cultures (Figure 5.6). This is in stark contrast to a previous report (Tropepe et al., 2001) that direct fate specification of ES cells in serum-free conditions proceeds via a LIF-dependent primitive neural stem cell. This is hardly surprising for a number of reasons: Firstly, LIF receptor (LIFR) deficient embryos, in which all LIF signalling is abolished, show no defect in early neural development (Li et al., 1995). Secondly, LIF has an established role as a self-renewal factor for ES cells, acting via the activation of the transcription factor STAT3 (Matsuda et al., 1999; Niwa et al., 1998; Smith et al., 1988; Williams et al., 1988). Moreover, in the experiments described by Tropepe *et al,* the majority of ES cells die in the first few hours after plating, suggesting that the effect of LIF is to enhance the viability of the ES cells rather than to promote the survival of a primitive neural stem cell. Other experiments have previously indicated that LIF suppresses ES cell differentiation in serum free conditions very similar to those reported by Tropepe *et al.* (Wiles and Johansson, 1999). The slight suppression of differentiation in the presence of LIF may be due to ES cell self renewal rather than alternative differentiation, as judged by morphology of cells in the cultures.

In the presence of serum, LIF is essential for ES cell maintenance. Withdrawal of LIF from ES cell cultures growing in the absence of feeders in conventional serum-containing medium causes rapid differentiation into mesoderm and endoderm (Smith, 1991). Therefore, routine culture of ES cells can be considered as a suppression of mesodermal and endodermal differentiation by LIF. The result discussed above, that ES cell differentiation in monolayer is only moderately inhibited by LIF indicates that this factor is not a potent inhibitor of neural differentiation. This implies that maintenance of ES cells by LIF requires the presence of a mesoderm/endoderm inducing signal and/or an independent activity to complement LIF.

I wanted to determine whether autocrine LIF signalling is a limiting factor for monolayer differentiation. Inhibition of LIF signalling by the LIF antagonist hLIF-05 did not increase the efficiency of Sox1 positive cell generation. The fact that the proportion of GFP positive cells decreased in the presence of hLIF-05 could be due

to reduced viability of ES cells in the absence of autocrine LIF signalling in the first few hours after plating in serum free medium.

Addition of exogenous FGF resulted in a slight reduction in the proportion of egfp positive cells (Figure 5.9). This contrasts with the previous report by Tropepe *et al,* which reports an increase in the number of neural colonies generated upon addition of FGF. However, as discussed previously, in the system used for their experiments, viability is compromised, so the effect of FGF might be to promote viability rather than neural specification. ES cells normally produce FGF-4 (Yuan et al., 1995). In our protocol, viability is high and the FGF needed for ES cell differentiation could be provided in an autocrine/paracrine fashion.

This is supported by the fact that FGF inhibition potently suppresses neural fate. This indicates that endogenous FGF signals are important either for the production of intermediate cells or the conversion of competent cells to *Sox1* positive ones. Tropepe *et al.* also report that inhibition of FGF signalling, either by blocking antibodies or using FGF receptor -/- cells, reduces the efficiency of the differentiation. These observations however are made on a very small proportion of initially plated cells and have little bearing on lineage specification. The reduction of efficiency observed in our system upon addition of high concentration of FGF could be because it stimulates proliferation of non-neural cells or because it is inducing mesoderm formation.

Inhibition of FGF signalling by the inhibitor SU5402 significantly reduced the appearance of *Sox1*^{egfp} expressing cells. This result indicates that endogenous FGF signals are important for neural specification of ES cells. A previously proposed action of FGFs in neural induction postulates a dual function: promotion of neural fate via an unknown mechanism and repression of *Bmp* gene expression. Co-addition of Noggin or Chordin to the SU5402 treated monolayers did not restore the efficiency of neural specification, indicating that the requirement for FGFs is not solely to repress *Bmp*s. This is consistent with experiments in the chick where strong inhibition of FGF signalling causes a repression of neural induction which cannot be reversed by BMP inhibition. However, in the same experiments, a lower dose of FGF inhibitor caused a Noggin-reversible repression of neural fates. My results failed to detect an effect on neural induction with lower doses of the FGF inhibitor.

6 Discussion

6.1 Targeting Sox1

The aim of the project was to create a reporter cell line to study neural differentiation of ES cells. The gene selected for the insertion of the reporter was *Sox1* because of its restricted expression during development and the fact that it is not expressed by ES cells. The targeting experiment resulted in the replacement of all of the *Sox1* open reading frame by the dual reporter/selection cassette EGFP IRES *pac*. Selection of transfected ES cells was achieved by virtue of a CMV hyTK cassette flanked by lox511 sites. After identification of correctly targeted clones by Southern hybridisation, selected clones were subjected to differentiation and analysed by flow cytometry for green fluorescence. No fluorescent cells were visible at that stage. The clones were subsequently transiently transfected with a Cre expressing plasmid to remove the CMV hyTK cassette. Recombined subclones were selected in the presence of ganciclovir and screened by Southern blotting. One clone was obtained initially and further examined, although three others were subsequently isolated and exhibited similar expression. This clone (46C) upon embryoid body neural differentiation upregulated the Sox1-GFP reporter and EGFP positive cells emerged.

The reason for the lack of fluorescence upon *in vitro* differentiation before the excision of the selection cassette remains unknown, but this phenomenon has been documented before (Faust *et al.*, 2000). A possible explanation is that the presence of a strong promoter (such as the CMV promoter) recruits transcriptional machinery from a nearby weaker promoter (*Sox1*) resulting in lower levels of expression from that promoter. Another possible explanation is that the presence of the CMV hyTK cassette 3′ to the *Sox1* ORF acts as a spacer separating regulatory sequences present in the 3′ region of *Sox1* from the promoter. This could result in reduced efficiency of the promoter.

The previous report on Sox1 targeting also describes lack of the reporter gene activity after insertion into the *Sox1* ORF (Nishiguchi et al., 1998). The authors hypothesise that this is due to the fact that "translation does not initiate from the translation start of βgeo" (the reporter was fused to the first 56 amino acids of Sox1). An alternative explanation suggested by my data is that the presence of the

MC1 *neo* cassette 3' of the reporter (used for selection in ES cells) competing with the endogenous promoter for transcriptional machinery, or the presence of the MC1 neo sequences separating promoter from regulatory/enhancer sequences is the reason.

Germline transmission of the targeted allele was accomplished only after the deletion of the selection cassette. Despite extensive breeding of chimeras generated from the original ES cell clones before the Cre-mediated removal of the CMV hyTK cassette, no germline transmission was observed. It is known that male transgenic animals carrying the TK gene rarely transmit it to their offspring. This is because a cryptic promoter sequence within the TK gene causes ectopic expression of a truncated product in mouse testis which in turn causes reduced fertility of the sperm (Ellison et al., 2000). The results obtained here indicate that the property of the TK gene to cause reduced fertility is maintained in its fusion with the *hph* gene encoding for hygromycin phosphotransferase.

The targeting efficiencies with the two vectors described in chapter 3 were very similar (2-3%). This was expected, as the homology arms used for their construction and the ES selection marker were identical. The main parameters that influence the targeting frequency are the length of the homology arms and the use of isogenic DNA, i.e. DNA derived from the same strain of mouse as the cells to be targeted. In both targeting experiments, the same cell line was targeted with identical regions of homology derived from isogenic DNA. Unfortunately no direct comparison can be drawn between this and a previous report on Sox1 targeting (Nishiguchi et al., 1998) because the frequency of targeting for those experiments is not clearly stated. These authors describe that 3 correctly targeted clones were injected into blastocysts and passed trough the germline, out of 465 screened. This implies that the targeting frequency was at least 0.65% if the only correctly targeted clones were those three. The total homology was smaller in those experiments, especially at the 3' of the ORF, which approached the 1Kb shown to be a minimum requirement for efficient recombination.

Heterozygous animals were generated on an outbred background and appeared phenotypically normal. Homozygous null animals for *Sox1* on the same background were obtained, but pups appeared smaller than their littermates. They also had smaller eyes with opaque lenses and appeared to be trembling. Adult -/-

animals had a more curved stature compared to their littermates, as well as smaller eyes and opaque lenses. Such homozygous null animals also suffered from sporadic fits, which caused them to roll over repeatedly for several minutes. The previously reported knockout for *Sox1* has the same phenotype on both inbred (129) and outbred (MF1) background. The cause of the epileptic fits has not been described in detail, but it appears they originate from the olfactory tubercle, which is missing from the Sox1 null animals (V. Episkopou, personal communication).

6.2 Sox1-GFP expression during development

Expression of the reporter construct was followed during mouse development and found to correlate well with previously published accounts. The onset of GFP fluorescence was found to be later than the onset of Sox1 expression determined by RNA hybridisation or immunofluorescence. This can be explained by two facts: Firstly, EGFP is a protein that needs to be correctly folded in order to fluoresce, and correct folding of it may take a few hours. Secondly, the concentration of EGFP molecules in the cytoplasm needed to confer fluorescence is limiting. Both *in situ* hybridisation and immunofluorescence employ the use of an antibody to detect the primary signal. This step can amplify the intensity of a weak signal, as in the case of low mRNA or protein levels. EGFP fluorescence is not amplified, so low levels of expression may pass undetected.

Sox1 expression has previously been documented to be associated with proliferating cells of the CNS (Pevny et al., 1998). This was not directly tested in my experiments, but the sites where Sox-egfp fluorescence was observed were areas of proliferation. In the early neural tube the majority of cells are dividing precursors with the exception of roofplate and floorplate cells which exit the cell cycle and participate in patterning the precursor cells of the neural tube along the dorso-ventral axis. As the neural tube thickens with continuing division of progenitor cells, cells start to differentiate and migrate outwards to populate the outer layers of the neural tube initially with neurons and later with glia. Fluorescence at these later stages becomes confined to the cells around the ventricular surface of the neural tube, which continue to proliferate. In the developing and adult brain expression is seen in regions known to contain proliferative cells, such as the telencephalic

vesicle, the subventricular zone of the lateral ventricles and the rostral migratory stream of migrating neuroblasts.

6.3 Monolayer differentiation and the default model

Monolayer differentiation resembles the default model for neural induction. Multipotent ES cells, resembling in their pluripotency primitive ectoderm cells of the mouse blastocyst, convert efficiently in the absence of any exogenous stimuli to neuroectodermal cells. This lineage restriction is blocked by bone morphogenetic protein, as is the neural differentiation of frog naïve ectoderm. ES cells express both BMPs and BMP receptor RNAs, but the level of endogenous BMP signalling does not appear to inhibit their differentiation under monolayer differentiation conditions. Addition of exogenous BMP antagonist did not enhance the differentiation efficiency, but could completely reverse the repression caused by exogenous BMP.

A difference between the effect of BMP during monolayer differentiation and during neural induction in frog is that in the ES cell system BMPs seem to promote mesodermal fates. Frog animal ectoderm cells acquire an epidermal fate if BMP signals are present, and a neural fate upon BMP inhibition. In the frog animal cap assay, whole explants cannot acquire neural fate without antagonism of endogenous BMP activity, but dissociation is sufficient to dilute out these signals and allow neural determination (Grunz and Tacke, 1989). This apparent discrepancy can be explained by different level of BMPs or their receptors being expressed in mouse ES cells to frog animal caps, or by the fact that ES cells express high levels of the BMP antagonist Follistatin and lower levels of the BMP antagonist Noggin (QiLong Ying, unpublished). Activity of these endogenous antagonists may be enough to counteract the effect of endogenous BMPs in these relatively low density cultures.

Analysis of the effect of other factors implicated in neural determination established a requirement for FGF signalling during monolayer differentiation. Pharmacological inhibition of signalling through FGFR caused a sharp reduction in the proportion of neural cells generated. This finding is in agreement with a proposed role of FGFs in neural determination (Wilson and Edlund, 2001). However, addition of exogenous FGF2 did not increase the proportion of Sox1-EGFP cells generated. This effect may be explained by the fact that ES cells express high levels

of FGF4. It is possible that autocrine FGF4 is sufficient to convert ES cells to neural cells, and exogenous FGFs only enhance proliferation of both neural and non-neural cells generated in the cultures, without skewing their relative proportions.

It has been proposed that the action of FGFs in neural induction is double: low levels of FGF signals act via an unknown pathway to promote neural fate, while high levels repress transcription of *Bmp*, indirectly promoting neural over non-neural fate. At a level determined to be sufficient to repress neural differentiation, the effect of FGF inhibition cannot be reversed by co-addition of Noggin, suggesting that the function of FGF is not simply to repress endogenous BMP levels. Such an action cannot be excluded however on these results, due to the expression of BMP antagonists by ES cells mentioned above. Furthermore, members of the FGF family are being expressed before and during mouse gastrulation and neural induction (Hebert *et al.*, 1991; Niswander and Martin, 1992), so a role for FGFs in mammalian neural induction cannot be precluded without further studies.

An interesting feature of monolayer differentiation is the time required for conversion of ES cells to neural precursors. 4 days after initiation of the differentiation, the vast majority of 46C cells express EGFP, indicating a conversion to the neural lineage. *In vivo*, ES cells are thought to correspond to the post implantation epiblast, around 4 days of development. The neural plate stage is around E7.5, and Sox1 expression as judged by EGFP expression in the animals generated from the 46C cells starts at E8.5. Therefore, the timing of neural determination of ES cells during monolayer differentiation is very similar to the timing of generation of Sox1 positive cells in the neural plate from the post-implantation epiblast. This timing similarity is seen in frog animal cap explants. Animal cap explants are derived from stage 10+ (around 10h post fertilisation) *Xenopus* embryos. When dissociated for more than 5 hours, they convert to neural cells equivalent to stage 14 (around 16h post fertilisation). This common aspect of the two *in vitro* differentiation systems may be an indication of a conserved temporal programme during these stages of development, controlling certain differentiation events.

The facts that the initiation of neural determination during monolayer differentiation is asynchronous between colonies, and that none of the factors tested could consistently increase the proportion of neural cells above that of

control untreated cultures prompt some questions. Is the process of neural determination stochastic, or governed by some community effect? Is the cell microenvironment providing a cue for neural determination to start, and what could the nature of that cue be? Is there an unknown factor produced by the cells at a limiting concentration required for neural determination? To address these points, more experiments need to be done with addition of factors during shorter periods of the differentiation process, dissociation of differentiating colonies before the onset of Sox1 expression and timelapse observation of the differentiation process.

6.4 Summary

This work has succeeded in generating, using gene targeting, a *Sox1* reporter cell line (46C). The allele generated is null and homozygous animals generated have a phenotype indistinguishable from the previously reported phenotype for *Sox1* null animals. Expression of the egfp reporter during development closely follows the expression of the *Sox1* gene and can be used to visualise proliferating precursors. The 46C cell line was used to analyse the conditions necessary for neural determination of ES cells in defined culture medium and on adherent monoculture. The results indicate that ES cells enter the neural fate upon liberation from self-renewal signals efficiently, and that this transition is dependent on endogenous FGF signals. Although BMP signals potently suppress this neural determination, inhibition of BMP either by antagonists or at the transcriptional level by FGFs does not appear to be required for it, contrary to the default model for neural induction. The mode of action of FGF to promote neural fate determination however remains to be elucidated.

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